Dynamics of individual sarcomeres during and after stretch in activated single myofibrils

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It is generally assumed that sarcomere lengths (SLs) change in isometric fibres following activation and following stretch on the descending limb of the force–length relationship, because of an inherent instability. Although this assumption has never been tested directly, instability and SL non-uniformity have been associated with several mechanical properties, such as ‘creep’ and force enhancement. The aim of this study was to test directly the hypothesis that sarcomeres are unstable on the descending limb of the force–length relationship. We used single myofibrils, isolated from rabbit psoas, that were attached to glass needles that allowed for controlled stretching of myofibrils. Images of the sarcomere striation pattern were projected onto a linear photodiode array, which was scanned at 20 Hz to produce dark–light patterns corresponding to the A- and I-bands, respectively. Starting from a mean SL of $2.55 \pm 0.07 \mu\text{m}$, stretches of $11.2 \pm 1.6\%$ of SL at a speed of $118.9 \pm 5.9 \text{ nm s}^{-1}$ were applied to the activated myofibrils ($\text{pCa}^{2+} = 4.75$). SLs along the myofibril were non-uniform before, during and after the stretch, but with few exceptions, they remained constant during the isometric period before stretch, and during the extended isometric period after stretch. Sarcomeres never lengthened to a point beyond thick and thin filament overlap. We conclude that sarcomeres are non-uniform but generally stable on the descending limb of the force–length relationship.

Keywords: instability; sarcomere length non-uniformity; force–length relationship; sarcomere force; sarcomere activation

1. INTRODUCTION

When a muscle fibre is activated on the descending limb of the force–length relationship, force has been found to ‘creep’, i.e. force continuously increases at a slow rate (e.g. Gordon *et al.* 1966; Edman & Reggiani 1984). Also, when a muscle or single fibre is stretched on the descending limb of the force–length relationship, the steady-state force following stretch is greater than the corresponding force obtained in a purely isometric contraction (Abbott & Aubert 1952; Edman *et al.* 1978, 1982; Julian & Morgan 1979a; Sugi & Tsuchiya 1988; Edman & Tsuchiya 1996; Linari *et al.* 2000; Morgan *et al.* 2000; Herzog & Leonard 2002). This phenomenon is called force enhancement following stretch. Creep and force enhancement have typically been assumed to be caused by the development of non-uniformities in sarcomere lengths (SLs) (Julian & Morgan 1979a; Morgan 1990, 1994). These non-uniformities in SLs were thought to occur on the descending limb of the force–length relationship exclusively, because of its presumed instability and the associated negative slope (Hill 1953).

Theoretical predictions based on an unstable descending limb of the force–length relationship show that, in a fixed-end contraction, only one sarcomere can reside on the descending limb: the remaining sarcomeres are predicted to either shorten onto the ascending limb of the force–length relationship, or elongate until they reach a positive passive force–length slope (Allinger *et al.* 1996; Zahalak 1997). However, there is no direct experimental

evidence for the development of SL non-uniformities and instability in a preparation in which each and all SLs were observed continuously, and furthermore, the theoretical predictions of sarcomere instability have typically not been observed experimentally.

Creep and force enhancement, and the corresponding issue of sarcomere instability, have played a significant role in muscle mechanics. Therefore, it is surprising that no systematic efforts have been devoted to investigate this issue in a preparation in which all SLs can be recorded continuously while activated and stretched on the descending limb of the force–length relationship. With the development of techniques to study single myofibrils, and the possibility to track the lengths of each sarcomere continuously (Bartoo *et al.* 1993, 1997; Blyakhman *et al.* 2001), the issue of SL non-uniformity and instability can be addressed directly. We used this approach to measure the length changes of all sarcomeres on the descending limb of the force–length relationship during and after stretch of activated myofibrils. In accordance with the idea of instability, we proposed that upon activation and following stretch on the descending limb of the force–length relationship, individual sarcomeres would continuously diverge, i.e. some sarcomeres would continuously elongate at the expense of other sarcomeres that would continuously shorten.

2. MATERIAL AND METHODS

(a) Specimens

Small strips of rabbit psoas muscle were carefully dissected and tied to small wooden sticks. These samples were stored in rigor solution (see § 2b) for 12 h. The specimens were then

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Figure 1. Schematic drawing of the apparatus used for the experiments. CCD, charge-coupled device.

transferred to a rigor : glycerol (50 : 50) solution for 12 h. Both procedures were carried out on ice throughout. After a new 12 h period, the specimens were transferred to a fresh rigor : glycerol (50 : 50) solution and stored in a freezer at -20°C for 7–15 days. On the day of the experiment, the muscle strips were placed in a rigor solution for at least 1 h before use. Small pieces of muscle tissue (*ca.* 2 mm length) were cut using a fine razor blade, and subsequently blended (Sorvall Omni Mixer) in rigor solution using the following sequence: twice for 5 s at 1100 r.p.m., twice for 1 s at 1800 r.p.m., once for 1 s at 2500 r.p.m., and once for 1 s at 3100 r.p.m.

(b) Solutions

The rigor solution (pH 7.4) was composed of (in mM): 50 Tris, 100 NaCl, 2 KCl, 2 MgCl_2 and 10 EGTA. The following protease inhibitors were added to the solution (in μM): 10 leupeptin, 5 pepstatin A, 0.2 phenylmethylsulphonyl fluoride, 0.5 NaN_3 and 0.5 dithiothreitol. The relaxing solution (pH = 7.0; $\text{pCa}^{2+} = 8$) was composed of (in mM): 10 MOPS, 64.4 K^+ propionate, 5.23 Mg^{2+} propionate, 9.45 Na_2SO_4 , 10 EGTA, 7 ATP, 10 creatine phosphate. The activating solution (pH = 7.0; $\text{pCa}^{2+} = 4.75$) was composed of (in mM): 10 MOPS, 45.1 K^+ propionate, 5.21 Mg^{2+} propionate, 9.27 Na_2SO_4 , 10 EGTA, 7.18 ATP, 10 creatine phosphate. All experiments were performed at room temperature ($20\text{--}22^{\circ}\text{C}$).

(c) Sarcomere length measurements

The apparatus used for force and SL measurements is shown schematically in figure 1. A small amount of blended muscle mixture was placed in a chamber whose bottom was made of a glass cover-slip. The chamber was positioned on top of a movable stage mounted on an inverted microscope (Zeiss, Axiovert 35, Germany). After allowing 5 min for stabilization, some myofibrils settled on the bottom of the chamber, while most myofibrils remained in suspension. The rigor solution was then slowly replaced with the relaxing solution, and the myofibrils in suspension were washed away, allowing for better visualization

of the undisturbed myofibrils positioned at the bottom of the chamber.

Myofibrils ($n = 8$) were attached to two glass needles that could be moved independently by two micromanipulators (figure 1). One glass needle was connected to a motor arm that allowed for fine displacement of the needle, and therefore precise changes in myofibril length. Under low magnification, the glass needles were centred in the optical field, and the microscope's phase-contrast system was adjusted. Under high magnification, provided by an oil-immersion phase-contrast lens (Zeiss, $\times 100$, numerical aperture 1.3), a myofibril was chosen for experimentation, based on its appearance and striation pattern.

The image of the myofibril was projected onto a linear, 1024-element photodiode array (Reticon, Santa Clara, CA, USA), which was scanned (20 Hz) to produce tracings of intensity versus position along the myofibril. Because of the contrast between dark (A-band) and light (I-band) regions, the photodiode array output generates a signal representing the sarcomere-banding pattern. SL was calculated by a minimum average risk algorithm method, based on the span between A-band centroids (figure 2). This system can detect SL changes with an accuracy of 2 nm (Blyakhman *et al.* 2001).

(d) (In)stability

Nominally, sarcomeres were defined as stable if their lengths remained constant, and were defined as unstable if their lengths changed unidirectionally (either shortening or lengthening, but not both) while the myofibril was held at a constant (isometric) length. Practically, because of potential noise in the SL traces, the following criteria were made to define a sarcomere as stable: (i) the range of length change (i.e. the difference between the longest and shortest SL) must be smaller than $0.1\ \mu\text{m}$ (*ca.* 4% of any SL encountered); and (ii) length changes for a given sarcomere must be random, i.e. shortening and lengthening phases during the isometric contraction of the myofibril. If a sarcomere had a maximal excursion of more than $0.1\ \mu\text{m}$, or if it changed

Figure 2. Procedures used to analyse sarcomere length. (a) Phase-contrast image of a myofibril. (b) Intensity peaks collected from the linear photodiode array. (c) Scans of the striation pattern of a myofibril during an imposed stretch. The span between the A-band centroids is defined as the sarcomere length (SL).

length unidirectionally (i.e. continuous shortening or continuous lengthening), the sarcomere was designated as unstable.

(e) Experimental protocol

Once a myofibril was set up for mechanical testing, and a clear striation pattern was obtained, a 10 min rest period was given. Then, the relaxing solution was replaced by the activating solution, and the myofibril contracted. After full activation, starting from a mean SL of *ca.* 2.55 μm , stretches of a nominal amplitude of 4–10% of myofibril length (actual, 4.8–17.0% of SL) were applied, with a nominal velocity of 100 nm s^{-1} (actual, $118.9 \pm 15.9 \text{ nm s}^{-1}$).

The lengths of the thick and thin filaments of rabbit skeletal muscle are 1.63 μm and 1.12 μm , respectively (Sosa *et al.* 1994), and the width of the bare zone is 0.15 μm (Squire 1981). Therefore, the descending limb of the force–SL relationship begins at 2.39 μm and extends to 3.87 μm . All stretches were initiated at average SLs along the descending limb of the force–length relationship.

3. RESULTS

Upon activation, but before stretching, sarcomeres had non-uniform lengths, but remained at a constant length (figure 3; 0–2 s). During stretching of the active myofibril, all sarcomeres ($n = 14$; figure 3; 2–5 s) elongated, albeit to a different degree. During the isometric phase following myofibril stretch (figure 3; last 8 s), individual SL changes were smaller than 0.1 μm , and any small length changes

were random and contained shortening and stretching phases. Therefore these sarcomeres were considered stable. The mean maximal dispersion of all sarcomeres in this myofibril was $0.016 \pm 0.001 \mu\text{m}$.

In two out of the eight tested myofibrils, two or three sarcomeres changed length in a directional manner and they were classified as unstable. Figure 4*a* shows one of these two examples, in which two sarcomeres (arrows) changed length directionally by 0.153 μm and 0.167 μm , respectively, during the 8 s isometric period following stretch. These two sarcomeres were adjacent to one another, they were next to the attachment point of the myofibril on the glass needle, and their combined SL change would have been classified as stable, with a maximal non-directional length change of 0.0285 μm (figure 4*b*).

Table 1 shows results from the eight myofibrils. SLs in all myofibrils were non-uniform upon activation. When the myofibrils were stretched, all sarcomeres elongated, albeit by different amounts. After the stretch, 105 out of 110 observed sarcomeres remained at nearly constant length; that is, length changes were randomly bi-directional and their maximal excursion was less than 0.1 μm . We found evidence of slow, systematic lengthening or shortening in five sarcomeres (two and three sarcomeres in two different myofibrils). These sarcomeres were always next to each other and next to the attachment of the myofibril to the glass needle.

From the 110 sarcomeres recorded in this study, none

Figure 3. (a) Length-time histories of all individual sarcomeres of a single activated myofibril during an isometric-stretch-isometric test. The initial average SL was $2.38\ \mu\text{m}$, and the average SL following stretch was $2.70\ \mu\text{m}$. Velocity of stretch: $107.5\ \text{nm s}^{-1}$. (b) Time-length traces during the experiment.

stretched beyond myofilament overlap (i.e. $\text{SL} > 3.87\ \mu\text{m}$). Three sarcomeres stretched to lengths of $3.34\ \mu\text{m}$, $3.33\ \mu\text{m}$ and $3.27\ \mu\text{m}$, respectively, each from a different myofibril. The remaining 107 sarcomeres did not stretch beyond $3.22\ \mu\text{m}$. In the range of SLs between $3.2\ \mu\text{m}$ and $3.4\ \mu\text{m}$, the passive force contribution to the total force in rabbit psoas myofibrils is small: *ca.* 5% of the active tension at full overlap of thick and thin filaments (Bartoo *et al.* 1993).

4. DISCUSSION

The main finding of this study was that activated sarcomeres on the descending limb of the force-length relationship are typically non-uniform in length but stable; that is, during isometric contraction of the myofibril, SLs remained virtually constant, and the minute length variations observed were small, random and bi-directional. This was true upon activation, and during the isometric period following myofibril stretch. Although the average SL during activation and length changes have been measured in several studies using single fibres (e.g. Ter Keurs *et al.* 1978; Julian & Morgan 1979*a,b*; Edman & Reggiani 1984, 1987), in this study we evaluated the length changes of each and all sarcomeres during and after stretch in single myofibrils.

Directional SL changes were observed in only two groups of sarcomeres. We believe that these directional length changes were artefacts of our technique for the following reasons. (i) They were only observed next to the attachment sites of the myofibrils. Attachment of the myofibrils involves a piercing of the needle tip into the myofibril, thus possibly causing some mechanical damage

Figure 4. Behaviour of individual sarcomeres during stretch in another activated myofibril. The thick line represents the average SL. The initial average SL was $2.43\ \mu\text{m}$, and the average SL following stretch was $2.81\ \mu\text{m}$. Velocity of stretch: $128.1\ \text{nm s}^{-1}$. (b) Same myofibril as shown in (a), but in this case only the two sarcomeres that change length following stretch are plotted. Note that these two sarcomeres are adjacent to each other and are at the end of the myofibril, right next to the attachment to the glass needle. Note further that the average of these two SLs is constant, suggesting that the apparent SL changes are caused by a single A-band shift in a damaged sarcomere. (c) Time-length traces during the experiment.

that might affect the stability of the thick filaments in the sarcomeres. (ii) Progressive length changes were local events involving two or three adjacent sarcomeres with no measurable influence on remote sarcomeres; thus damage of a sarcomere at the attachment site was contained locally. (iii) The combined length changes of two sets of 'unstable' sarcomeres gave a constant total length change (figure 4b), suggesting that the instability was produced by the shift of one (two sarcomeres) or two (three sarcomeres) A-bands from the centre of a damaged sarcomere. Despite the great range of individual SLs before and after stretch (figures 3 and 4), it appears that 105 out of 110 measured sarcomeres from eight myofibrils were perfectly stable on the descending limb of the force-length relationship.

Our findings are in contrast to the theoretically predicted unstable SL behaviour on the descending limb of

Table 1. Behaviour of all sarcomeres investigated in eight myofibrils.

myofibril	n	sarcomeres		
		stable	unstable	maximal dispersion (μm)
1	16	16	0	0.017 ± 0.002
2	12	12	0	0.033 ± 0.004
3	11	11	0	0.047 ± 0.015
4	12	12	0	0.046 ± 0.009
5	14	14	0	0.016 ± 0.001
6	30	30	0	0.045 ± 0.004
7	7	5	2 ^a	0.075 ± 0.022
8	8	5	3 ^a	0.109 ± 0.042

^a Adjacent to each other and next to the attachment of the myofibril to the glass needle.

the force-length relationship (e.g. Morgan 1990, 1994; Zahalak 1997), a concept introduced almost half a century ago by Hill (1953). The results also do not agree with the notion of 'popped' sarcomeres that are supposed to occur immediately (1–2 s) following stretch of single fibres on the descending limb of the force-length relationship (Morgan 1994).

The notion of instability, and the corresponding continuous and uni-directional divergence of SLs, has been used to explain the steady-state force enhancement observed after active stretch of muscles and fibres (Julian & Morgan 1979a; Morgan 1994; Morgan *et al.* 2000), as well as the force creep observed in some muscle and fibre preparations (e.g. Gordon *et al.* 1966; Edman & Reggiani 1984). Julian & Morgan (1979a) observed that during stretch of activated muscle fibres, sarcomeres did not stretch by the same amount. Sarcomeres toward the ends of the fibre stretched less than average, while sarcomeres near the centre stretched more than average. Our observations on single myofibrils support the results of Julian & Morgan (1979a). However, they cannot be explained with the idea of instability on the descending limb of the force-length relationship because sarcomeres did not exhibit continuous uni-directional length changes during the isometric phase of myofibril contraction, as expected in an unstable system (Allinger *et al.* 1996; Zahalak 1997).

Our results on SL non-uniformity are qualitatively similar to those of Edman *et al.* (1982), who observed that all sections of isolated muscle fibre were elongated during fibre stretch, and that the different segments were never stretched beyond filament overlap. The advantage of using isolated myofibrils, rather than single fibres, is that the lengths of all sarcomeres can be measured and analysed individually and continuously.

One of the limitations of this study is that we did not measure force simultaneously with SL in the myofibrils. However, we performed force measurements in five additional myofibrils that were actively stretched on the descending limb of the force-length relationship. In these additional pilot experiments, we observed a substantial amount of force enhancement (D. E. Rassier, W. Herzog and G. H. Pollack, unpublished observations), similar to what has been observed in single muscle fibres (e.g.

Julian & Morgan 1979a; Edman *et al.* 1982; Edman & Tsuchiya 1996) and whole muscles (e.g. Morgan *et al.* 2000; Herzog & Leonard 2002). These preliminary results suggest that force enhancement after stretch is also a property observed in isolated myofibrils.

We obtained two basic results in this study: SLs are non-uniform, and sarcomeres are stable on the descending limb of the force-length relationship. In a myofibril, sarcomeres are arranged strictly in series. Therefore, the force exerted by all sarcomeres in steady-state configurations, as observed here, must be the same. One of the most basic principles of the cross-bridge theory of muscle contraction is that the isometric, steady-state force in each sarcomere on the descending limb of the force-length relationship is given by actin-myosin overlap (i.e. by SL). Here, this principle was violated. For example, in figure 3, we show a myofibril with 14 sarcomeres in series, and each of the sarcomeres behaves in a perfectly stable manner following stretching on the descending limb of the force-length relationship. However, following stretch, the shortest sarcomere is *ca.* 2.4 μm and the longest sarcomere is greater than 3.0 μm . The basic question that arises from this result is: how can sarcomeres that are in series with each other and are 2.4 μm and 3.0 μm in length be stable and at force equilibrium?

Several possibilities exist to explain this result. The sarcomere at 3.0 μm (figure 3) may have more contractile proteins (actin and myosin filaments) than the sarcomere at 2.4 μm ; thus its smaller overlap compared with the remaining sarcomeres might be compensated for by an increased amount of contractile proteins. However, if this possibility were correct, two sarcomeres of similar (equal) length prior to stretching should also be of similar (equal) length after stretching. Figure 3 reveals that this is not the case. Another possibility is that individual sarcomeres have different passive properties, and a lack of active force in a long sarcomere might be compensated for by the corresponding passive force. However, the passive forces in the myofibril shown in figure 3 are probably less than 5% of the maximal isometric force (Bartoo *et al.* 1993), whereas the difference in active force for sarcomeres at 2.4 μm and 3.0 μm should be *ca.* 40% of the maximal isometric force (Gordon *et al.* 1966), a discrepancy that seems too big to be accounted for by a difference in passive force. Therefore, it appears that sarcomeres at vastly different lengths on the descending limb of the force-length relationship can produce the same force. It remains to be seen how this surprising result, which was found in all myofibrils, can be explained. In the meantime, the current results stand as a challenge to the idea that sarcomeres are unstable on the descending limb of the force-length relationship.

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