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THE EFFECT OF TENDON COMPLIANCE ON *IN VITRO*/*IN VIVO* ESTIMATIONS OF SARCOMERE LENGTH

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Summary

The errors likely to result from using excised rigor muscles to determine *in vivo* sarcomere length ranges were calculated for mouse extensor digitorum longus muscle (EDL). This muscle was chosen because its very long tendon makes it particularly susceptible to errors arising from tendon compliance. By placing dissected limbs into different locomotory stances, and allowing them to go into rigor, the range of sarcomere lengths over which muscles operate *in vivo* can be determined, but it is subject to errors due to tendon compliance. A tendon compliance of 0.24 GPa and a muscle rigor stress of 35 kPa were determined, and these were used to correct the estimates of *in vivo* sarcomere length, under worst case conditions. The error introduced was very small: a reduction in sarcomere length of less than 0.5 %.

Key words: locomotion, mammals, muscle, sarcomere length, tendon, work loops, mouse.

Introduction

The work loop technique (Josephson, 1985) is used to simulate *in vitro* the conditions under which muscles operate *in vivo*. Most of this research is related to animal locomotion and to the operation of skeletal muscles. An important aspect of this work is the ability to relate the sarcomere length operating ranges *in vitro* to those *in vivo*. Estimates of *in vivo* muscle strains and sarcomere lengths have often been made from kinematic data (Dimery, 1985; Cutts, 1986, 1988; Rome and Sosnicki, 1991; James *et al.* 1995). Generally, high-speed video or cine film is used to determine limb positions during locomotion. The cadavers of freshly killed animals are then positioned in such a way as to reproduce the attitudes observed during locomotion and are allowed to go into rigor. The muscles of interest are fixed by immersion, perfusion or injection of a fixative solution, usually formaldehyde or glutaraldehyde. Muscle fibres are then dissected out and sarcomere length determined microscopically or by light diffraction.

James *et al.* (1995) examined the properties of mouse fast twitch (extensor digitorum longus, EDL) and slow twitch (soleus) muscle using the work loop technique. They related the *in vitro* muscle performance under different strains, oscillatory cycle frequencies and stimulation regimes to the *in vivo* operation of the muscles, determined by kinematic analysis of high-speed video sequences of mice during locomotion. During locomotion, tendons will be stretched by the forces exerted upon them. These forces vary cyclically because of the cyclic activity of the muscles attached to them. The more compliant a tendon is, the more the muscle attached to it will need to shorten to produce a given action. When limbs are being positioned to reproduce the attitudes observed during locomotion, the tendons are under negligible stress. As *rigor mortis* develops, tendon stresses will increase. However, unless the stress attributable to *rigor mortis* is the same as the stresses encountered during locomotion, tendon strains, and therefore muscle and sarcomere lengths, will also be different. Locomotory stresses also vary throughout the muscle length change cycle. It is the aim of this paper to determine the error that could arise from measuring sarcomere lengths with this widely used technique.

We have measured muscle rigor stresses and compared them with maximum tetanic stress and with the stresses observed during work loop studies (e.g. Altringham and Young, 1991; James *et al.* 1995), probably the best estimate of the periodically varying stresses that occur during locomotion. We have also measured tendon compliance and tangential moduli using a range of loads which simulate these stresses. From a comparison of the relative tendon and muscle strains during rigor and during locomotion, we estimated the errors involved when sarcomere lengths were measured after fixation under rigor stress alone. Mouse extensor digitorum longus (EDL) was used, since this muscle has a very long tendon in most quadrupedal mammals, and would therefore be particularly prone to such errors.

Materials and methods

Determination of rigor stress

Mouse EDL muscle–tendon complexes were clamped on the tendons, using aluminium foil clips placed less than 1 mm

away from either end of the muscle. One end was attached to a micromanipulator and the other end to an isometric force transducer (Harvard). Muscle length was adjusted to the length for maximum isometric force production (L_0) by maximising the twitch force elicited by a single, supramaximal electrical stimulus. The muscles were then allowed to go into *rigor mortis* in non-oxygenated Ringer's solution (composition in mmol l^{-1}: NaCl, 144; KCl, 6; $MgCl_2$, 1; NaH_2PO_4, 1; $MgSO_4$, 1; Hepes, 10; $CaCl_2$, 2; pH 7.4 at 21 °C) at room temperature (20 °C). Muscle force was recorded continuously using a pen-recorder.

Determination of tendon stress

EDL muscle and tendon dimensions, mass, volume, cross-sectional area, total muscle and muscle fibre lengths were determined. Stress for each of the four distal tendons was calculated as the total muscle force divided by four, in turn divided by tendon cross-sectional area. The tendon cross-sectional areas were very similar, as were the muscle cross-sectional areas inserting into them; therefore, the four tendons were treated as a single unit in all calculations.

Determination of tendon compliance

One of the four distal EDL tendons was glued at both ends into aluminium foil clips using cyanoacrylate glue. Preliminary experiments showed that considerable stresses could be achieved using this system without substantial deformation of the foil clips or marked tendon slippage. They were attached at one end to a micromanipulator connected to a position transducer (Harvard) and at the other end to an isometric force transducer (Harvard). The tendon strain was then progressively increased by moving the micromanipulator. Force and length were recorded on a digital storage oscilloscope (Gould 1604). Care was taken to maintain the hydrated state of the tendon by irrigating with isotonic Ringer's solution. The resulting stress and strain were determined from the force and length records. In each calculation, the stresses were divided by four to account for there being four distal tendons (of similar cross-sectional area). Plots of stress *versus* strain yielded an essentially linear relationship over the range of stresses to which the tendons were subjected (see Fig. 1). This range included stresses equivalent to the mean rigor stress, maximum isometric stress and the predicted maximum locomotory stress. The material compliance of the tendon under this range of stresses was estimated as the gradient of a least-squares linear regression fitted to the stress–strain data.

Calculation of tendon strain and additional muscle shortening

EDL tendon stresses during locomotion were estimated using the pattern of muscle stresses observed during work loop studies (James *et al.* 1995). Tendon strain was then determined for each point in the cycle that the muscle was active, as the tendon stress divided by tangential modulus.

We have assumed that the proximal tendon has the same cross-sectional area as the distal tendon. In fact, the proximal tendon is thicker but only a fraction of the length of the distal tendon, so the errors involved in this assumption are small. We also assumed that, during operation at galloping frequencies, the muscle generates the same stress as it does *in vitro* during an optimal work loop cycle at the same cycle frequency (James *et al.* 1995). These assumptions represent a worst case condition that will tend to overestimate tendon stress and strain.

The total length of the muscle–tendon unit must follow the kinematically derived strain pattern. Therefore, extension of the tendons must be taken up by additional muscle shortening. If we assume that the muscle is parallel-fibred (as is essentially the case), and that there are no substantial heterogeneities in sarcomere length, the sarcomeres will shorten by the same proportion.

Results

Dimensions were: muscle length=10.9±0.1 mm (N=4), muscle fibre length=8.2±0.1 mm (N=4), proximal tendon length=2.3±0.2 mm (N=3), distal tendon length=16.7±0.7 mm (N=6), muscle fibre cross-sectional area=1.0±0.1 mm^2 (N=4), proximal tendon cross-sectional area=0.14±0.0 mm^2 (N=2) and distal tendon cross-sectional area=0.08±0.01 mm^2 (N=6). All values are mean ± S.E.M., N is the number of observations. A muscle rigor stress of 35.0±7.8 kPa (N=3) was obtained. A linear relationship was obtained between stress and strain over the stresses likely to occur during locomotion (Fig. 1), giving a tangential modulus of 0.24±0.006 GPa (N=2; in each case, r^2>0.96).

Fig. 2 shows that, when the extension due to tendon compliance is accounted for, the estimated sarcomere lengths in active muscle during locomotion (small filled circles) become shorter than those determined when the tendon is under rigor stress alone (large open circles). However, the strain pattern remains largely unaltered, the largest error being a reduction in sarcomere length of less than 0.01 μm (less than 0.5 % of mean sarcomere length). The corrected values fall on the solid line, which was determined from uncorrected data by five-point smoothing.

Discussion

The rigor stress obtained in this study was considerably lower than that measured by White (1970) for skinned rabbit psoas muscle fibre bundles. It is possible that not all of the fibres of a whole muscle achieve maximum rigor stress simultaneously (rigor developed over 8–10 h). The fibres which first achieve rigor may relax before further fibres begin to develop force. Consequently, a whole muscle may not reach the same maximum rigor stress as a single fibre or a small bundle of fibres.

The value for the tangential modulus is approximately half the value for Young's modulus estimated by extrapolation of data for large mammals from Bennett *et al.* (1986). This yields a value of 0.45 GPa for a hypothetical tendon under a stress

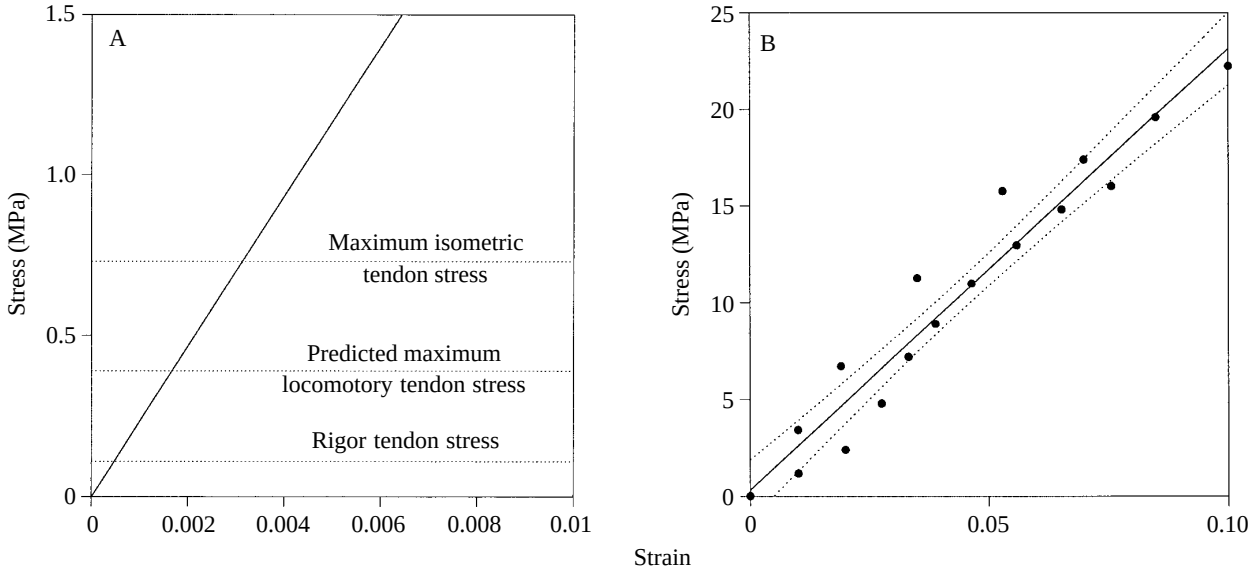

Fig. 1. (A) Stress *versus* strain relationship over the physiological range for two mouse EDL distal tendons. The maximum locomotory tendon stress is calculated using the peak stress shown in Fig. 2. Maximum isometric tendon stress is calculated from maximum isometric muscle stress (James *et al.* 1995). Tendon stress is calculated from muscle stress due to rigor. In each calculation, the stresses are divided by four to account for there being four distal tendons (of similar cross-sectional area). (B) Distal tendon stress *versus* strain relationship over the full range measured. The line represents a first-order polynomial fitted to the data using least-squares regression. r^2=0.94. Dotted lines show 95 % confidence limits.

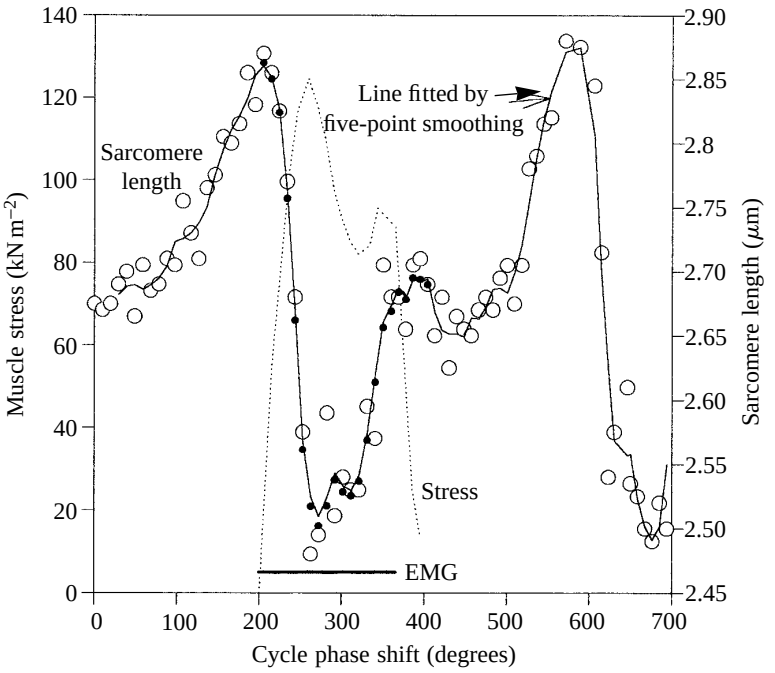

Fig. 2. Mouse EDL sarcomere lengths used during locomotion, taken from James *et al.* (1995), represented by large open circles. The solid line was derived from five-point smoothing of the uncorrected sarcomere length data. Smoothed sarcomere length data, after correction for tendon elasticity, are shown by the filled circles. The solid bar represents the EMG from the rat EDL muscle (Nicolopoulos-Stournaras and Iles, 1984). The muscle stress record (dotted line) was determined from work loop studies (James *et al.* 1995). Cycle phase shift: 0–360 ° represents one complete stride, starting from initial contact of the foot with the ground at 0 °.

similar to that measured for mouse EDL. The value for the elastic modulus obtained in this study, however, does fall within the range measured by Shadwick (1990) for newborn (0.16 GPa) to adult pig toe extensor tendons (0.76 GPa). If we use either of the higher values for Young's modulus (0.45 GPa from Bennett *et al.* 1986, or 0.76 GPa from Shadwick, 1990), instead of the modulus obtained in the present investigation (0.24 GPa), the estimate of tendon strain would be reduced.

The low values for the tendon tangential modulus obtained in this investigation are worthy of comment. No evidence of stress relaxation or of substantial slippage of the tendon within the clips (in the form of either gradual or sudden decreases in measured stress with increasing strain) was found over the range of stresses applied. If there had been errors due to either of these effects, our calculated value of compliance would have been artificially increased. This would have made our calculation of error in sarcomere length measurement an even greater overestimate. The low values for tendon tangential

moduli might, however, be expected. There is considerable variation in the values found for elastic modulus of tendons by different investigators. Bennett *et al.* (1986) found a range of moduli from 0.92 GPa in deer gastrocnemius to 2.00 GPa in dolphin tail tendons; all of their measurements were taken at stresses of greater than 30 MPa. The toe region of a stress–strain plot extends up to stresses of between 10 and 30 MPa. It is well recognised that stresses of greater than 30 MPa correspond to values in the linear region of a stress–strain plot and stress values considerably below that fall upon the toe region of a stress–strain plot. Shadwick's (1990) values for elastic modulus vary: new-born pig tendons, 0.16 GPa at stresses greater than 3 MPa; adult pig toe extensor tendons, 0.76 GPa at stresses greater than 10 MPa; and flexors, 1.66 GPa at stresses greater than 30 MPa. Our value for elastic modulus and Shadwick's lower values for elastic moduli almost certainly fall within the toe region of a stress–strain plot.

Ker *et al.* (1988) showed that tendons which act within the body as springs are attached to muscles with relatively short fibres, muscle-to-tendon cross-sectional area ratio is greater than 75 and *in vivo* stresses are greater than 25 MPa. In common with Ker *et al.* (1988) and Shadwick (1990), we find that the digital extensor tendons used in this study (EDL) have a low muscle-to-tendon cross-sectional area ratio (13) and low calculated peak locomotory stresses (approximately 5.4 MPa). Therefore, they would not seem to be acting as springs, but instead appear to act as a relatively inextensible link between muscle and bone. Biewener *et al.* (1981) also found that small mammals (kangaroo rat, 100 g) often have tendons which are far thicker than 'necessary'. They suggest that this may be due to a reduced importance of elastic storage in the terrestrial locomotion of small mammals. This conclusion is shared by Alexander *et al.* (1981).

James *et al.* (1995) used a combination of the work loop technique and a kinematic analysis of running mice to analyse muscle function in locomotion and included an estimate of the *in vivo* operating sarcomere length range of EDL. EMG records for the rat (Nicolopoulos-Stournaras and Iles, 1984) were used by to predict the period of the strain cycle over which the EDL is probably active.

We have made new calculations of sarcomere length correcting for tendon compliance, based on the following assumptions: (1) that the muscle is maximally activated throughout the period of EMG activity, (2) that the muscle develops the same stress at each estimated *in vivo* strain as it does during an *in vitro* work loop at the same frequency, and (3) that the work loop cycle has the same duration of shortening as the shortening period of the kinematically determined locomotory cycle.

These conditions maximise tendon strain and the period over which the tendon will be stretched. As can be seen from Fig. 2, correcting for tendon compliance results in shorter predicted sarcomere lengths when the tendon is under stress. However, the muscle length change pattern remains largely unaltered over most of the cycle. The greatest error of less than 0.01 μm (less than 0.5 % of the mean sarcomere length) occurs briefly at high stress and late in the muscle shortening phase. This error will have an insignificant effect on muscle function and power output. The error would still be less than 2 % of the mean sarcomere length at the greatest stress the muscle can sustain (approximately twice maximum isometric stress). We conclude that measurements of sarcomere length made upon muscle fixed during rigor provide a useful representation of muscle strain during locomotion, at least in small terrestrial mammals.

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