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Cross Bridge Slippage Induced by the ATP Analogue AMP-PNP and Stretch in Glycerol-Extracted Fibrillar Muscle Fibres

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Abstract. Glycerol-extracted insect fibrillar muscle fibres in rigor exhibited both an elastic and a plastic phase in the length-tension diagram. The transition between these phases took place at a critical tension, the "yield point" or elastic limit. In the plastic phase the apparent static elastic modulus became zero, whereas the immediate elastic modulus (measured by rapid length changes completed within 4 ms) exhibited no abrupt change at the yield point. The tension value of the yield point (but not immediate stiffness) was lowered by addition of AMP-PNP and was partially restored by washing out AMP-PNP. The dependence of the critical tension at which plastic flow begins on cooperative cross bridge behaviour is discussed in terms of breaking and reforming acto-myosin linkages. Evidence is presented that addition of AMP-PNP induces slippage of cross bridges on the actin filament by affecting the interaction between myosin and actin.

Key words: Muscle rigor – Cross bridge slippage – ATP analogue – Insect fibrillar muscle – Muscle stiffness.

Introduction

Glycerol-extracted fibre bundles immersed in a saline containing MgATP and EGTA (low $[Ca^{++}]$: $< 10^{-8}$ M) are in a relaxed state. When MgATP is washed out of the bath the fibre preparation contracts isometrically, entering a rigor state (cf. White, 1970). In the rigor state the immediate stiffness, i.e. the resistance to a quick stretch, is far greater than in the relaxed state. This change in mechanical behaviour has been attributed to structural differences between the two states: in rigor the cross bridges are attached to the actin filaments in an acute-angled conformation, while in the relaxed state they are detached (Reedy et al., 1965).

At tension values lower than the isometrically generated tension, the fibre preparation in rigor shows elastic behaviour: if the preparation is relaxed and then restretched to its original length (L_i), the tension within the preparation returns again to the value of the isometrically generated tension. When the rigor preparation is

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stretched to a length greater than L_l, this gives rise to a tension higher than the isometric tension but the preparation no longer behaves in an elastic fashion (cf. Maréchal, 1960).

The yield point is the critical tension at which the transition from elastic to plastic behaviour occurs. Addition of AMP-PNP (an ATP analogue which is not split by myosin; Yount et al., 1971) reduces isometric rigor tension without lowering immediate stiffness (Barrington Leigh et al., 1973; Kuhn, 1973; Beinbrech et al., 1976). There are at least two possible interpretations of this phenomenon when it is assumed that immediate stiffness is a measure for the number of cross bridges attached to the actin filament (cf. Huxley and Simmons, 1971). Firstly, a conformational change (rotation and/or bending) of cross bridges may occur while they remain attached to the actin filament and secondly there may be a change in the locus of attachment of cross bridges to the actin filament while the number of attached bridges remain constant (slippage). Electron micrographs (Beinbrech et al., 1976) have shown that addition of AMP-PNP to muscle fibres induces an increase in the number of cross bridges attached to the actin in a perpendicular position at the cost of the number of acute angled bridges. The results of x-ray diffraction studies (Marston et al., 1976; Goody et al., 1975; Lymn and Huxley, 1973) have been interpreted (cf. Goody et al., 1976) to imply that neither of the mechanisms mentioned above (slippage or conformational change of cross bridges) can alone explain the changes of diffraction pattern induced by addition of AMP-PNP. We decided to investigate the effect of AMP-PNP on the elastic limit (yield point) in order to find out whether alteration in the conformation of cross bridges is the only factor or whether there is also a change in the locus of attachment (i.e. a slippage) of the cross bridges.

Methods

Dorsal longitudinal muscle fibres from Lethocerus maximus were glycerol-extracted (cf. Jewell and Rüegg, 1966) and stored for up to 4 months in a 50/50 v/v glycerol: water mixture pH 7 at −16° C. Fibre bundles (5–10 fibres) were first relaxed in an ATP-salt solution (15 mM ATP; 15 mM $MgCl_2$; 10 mM NaN_3; 20 mM imidazole; 4 mM EGTA) at low Ca^{++} concentration and they contracted at high Ca^{++} concentration (obtained by adding 3.5 mM $CaCl_2$ to the relaxation solution).

Only fibres which relaxed in the absence of Ca ions and fibres which showed a marked delayed tension rise (Jewell and Rüegg, 1966) in the presence of Ca^{++} were used for further experimentation. Rigor contraction was induced following the procedure of White (1970):

The bundles were first incubated in relaxation solution and then immersed in an ATP-free rigor solution (60 mM KCl; 10 mM NaN_3; 20 mM imidazole; 4 mM EGTA; 5 mM $MgCl_2$). After addition of AMP-PNP (adenylyl-imidodiphosphate) and an equivalent amount of $MgCl_2$ the ionic strength was adjusted with KCl.

AMP-PNP (purchased from Boehringer, Mannheim) contained about 6% ADP as estimated from an enzymatic test when an ATP regenerating system and NADH was used. To prevent disproportionation of ADP into ATP and AMP by the myokinase still present in glycerol-extracted fibres (Abbott and Leech, 1973), the myokinase inhibitor P^1, P^5-di(adenosine-5′-)pentaphosphate (Feldhaus et al., 1975) pur-

chased from Boehringer, Mannheim was added to the AMP-PNP solutions in a final concentration of 0.2 mM.

Mechanical Measurements: For measurements of tension the bundles were glued between two glass rods. One glass rod was rigidly connected to a RCA 5734 transducer (resonance frequency 1.1 kHz). The force transducer was fixed on a micrometer drive the position of which could be monitored by the displacement of a Grass force transducer FTOC3. The second glass rod was connected to a Vibrator (Ling Dynamics 101) which could perform length changes within 4 ms. The position of this second glass rod was measured by field plates (Siemens, FP 17 L100). The baseline of force was checked when fibres in rigor were completely released by 3% L_i.

Immediate elastic modulus was measured when fast length steps (rise time 4 ms, duration up to 100 ms) of amplitudes $\pm$ 5 μm, $\pm$ 10 μm, $\pm$ 15 μm were applied to fibres of an initial length (L_i) which varied between 4.85 and 5.20 mm. The extreme tensions reached during the length change were then plotted as T_1 curves (cf. Huxley and Simmons, 1971) versus the axial strain component ($\varepsilon = \Delta L_i/L_i$). The T_1 curves could be fitted by a relation which depends parabolically on the axial strain component:

$$T_1 = T_0 + E\varepsilon\,(1 + E_2\varepsilon)\,,$$

where T_0 is the tension before the length change, E is immediate elastic modulus extrapolated to zero strain, and $E_2 = 30 \pm 10$ (SD, $n = 20$). E_2 is a measure of the deviation from Hooke's law of the observed elastic responses. The static elastic modulus ($E_{\text{stat.}}$) was calculated from the quotient of the tension (force per fibre) difference between the tension reached 5 min after a length change was performed and the initial tension before the length change divided by the relative length change ($E_{\text{stat.}} = \Delta T/\Delta L_i$; $\Delta L_i \leq 0.2\%\ L_i$).

Results

Glycerol-extracted bundles (5–8 fibres) from Lethocerus maximus were first immersed in 2 ml of a relaxing solution containing 15 mM MgATP, 4 mM EGTA, 20 mM imidazole, 1 mM NaN_3 and 10 mM KCl pH 6.7 at 12° C.

In the relaxed state tension was adjusted to 10 μN/fibre by variation of the initial length. The immediate elastic modulus of the ATP relaxed fibres was 5.5 mN/fibre. The MgATP was then removed from the fibres by transferring the bundle to rigor solution (60 mM KCl, 5 mM $MgCl_2$, 20 mM imidazole, 1 mM NaN_3, 4 mM EGTA pH 6.7, 12° C). After a typical delay time (cf. White, 1970) the bundles isometrically contracted to a high steady state tension (150–220 μN/fibre, $n = 8$) and to a high value of immediate elastic modulus (20–25 mN/fibre). The isometric tension was quite stable: in a prolonged experiment the levels of rigor tension were constant 180, 175, 170, 175 μN/fibre at 0.5, 6, 12, 24 h. When a fibre bundle was maintained for 24 h in released state (−1.2% L_i) tension was nearly constant 25, 30, 30, 25 μN/fibre at 0.5, 6, 12, 24 h after an initial recovery phase. Figure 1 shows a typical length-tension diagram of a bundle (6 fibres). Such behaviour was shown consistent-

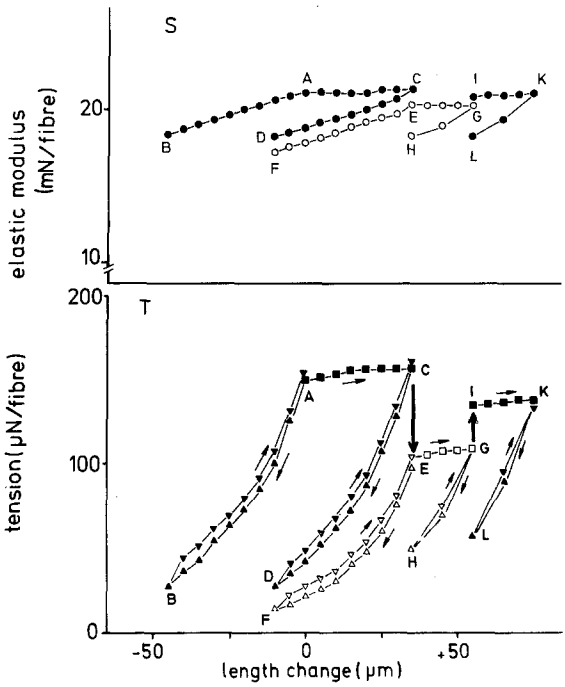

Fig. 1. S Plot of immediate stiffness versus length change for a bundle (6 fibres) of glycerol-extracted dorsal longitudinal muscle from Lethocerus maximus. **T** Length-tension diagram. Rigor tension was generated at *A* by removing the ATP from relaxed fibres at $\Delta L_i = 0$ (initial length $L_i = 5.05$ mm). Solid symbols refer to steady state tension levels and immediate stiffness values observed with fibres in rigor. Addition of 0.1 mM AMP-PNP induced an isometrical tension decrease (↓); open symbols refer to tension and immediate elastic modulus observed in the presence of AMP-PNP. When AMP-PNP was removed from the fibre, tension rose isometrically (↑). For further explanation, see text. Tensions: (▲, △) release; (▼, ▽) restretch; (■, □) stretch. Conditions: pH 6.7; I = 0.06 M; 12° C; 0.1 mM AMP-PNP

ly in a total of eight experiments. From initial rigor the bundle was first released by 0.2% L_i steps up to 1.2% L_i. Tension (▲) as well as immediate stiffness (●) was recorded 20 min after the length step was performed (*A*–*B*, Fig. 1). When tension is plotted against the length change a slightly curved plot results. Tension drops from 180 μN/fibre at $\Delta L_i = 0$ to 25 μN/fibre at $\Delta L_i = -1.2\%\ L_i$. Immediate elastic modulus also decreases from 22.5 to 18.4 mN/fibre upon this release.

The slope of the length-tension diagram gives the static stiffness of the fibre. The calculated values of the static elastic modulus values are much smaller than the immediate stiffness values: at $\Delta L_i = 0$ static elastic modulus was 14.3 mN/fibre whereas immediate elastic modulus was 22.5 mN/fibre. When the fibre bundle was restretched through the same sequence of length changes (*B*–*A*), tension (▼) and immediate elastic modulus (●) increased again. Only small hysteresis loops were observed at a given fibre length since tension levels (▲) recorded during release differ only slightly from tension levels (▼) observed during restretching. The reversibility of the length-tension plot *A*–*B*–*A* shows that the fibre in rigor behaves elastically upon release from initial tension.

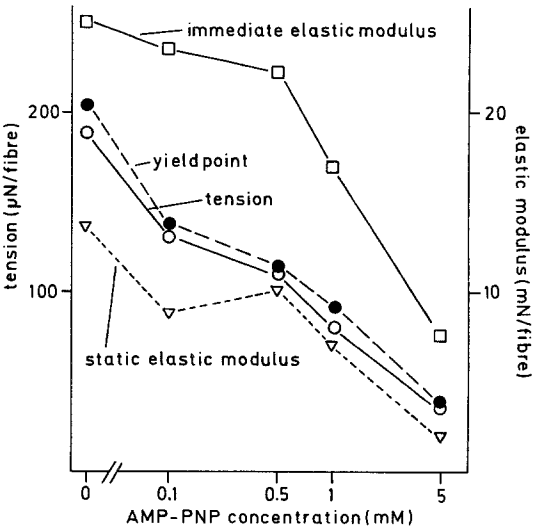

Fig. 2. Isometric tension (○) was generated for each AMP-PNP concentration when ATP was washed out from an ATP relaxed bundle (8 fibres) by immersing it in a saline containing the AMP-PNP concentration given on the abscissa. Yield point (●); immediate elastic modulus (□); static elastic modulus (▽). Conditions: bundle of 8 fibres; pH 6.7; I = 0.06 M; 12° C

Then the bundle was stretched by 0.2% L_i steps to lengths exceeding the initial length (A—C). The tension levels (■) and the values of immediate stiffness (●) (recorded again 20 min after the length change was performed) were now nearly equal to the tension levels before the stretch. This indicates that the fibre underwent plastic flow during this phase (A—C) of the experiment. Such a plastic stress-strain behaviour was observed whenever the fibre tension was greater than the initial rigor tension. The bundles always behaved elastically when they were released: the elastic trace (C—D) performed after the plastic flow (A—C) shows that upon release the elasticity is regained nearly completely.

Figure 1 shows further that addition of AMP-PNP (0.1 mM, C—E) induced isometric relaxation of the fibre bundle with practically constant immediate stiffness. Repeating the release stretch cycle [tension (△, ▽), immediate elastic modulus (○), E—F—E] then shows that fibres charged with AMP-PNP behave elastically at tension levels lower than the isometric tension levels characteristic for the AMP-PNP. Static fibre stiffness (estimated from the slope of the trace E—F) is smaller than static stiffness in rigor. The tension level at E seems to be a new yield point since further stretching of the fibre did not change the tension level in the steady state (E—G), whereas a subsequent release produced an elastic response (G—H). Adding AMP-PNP to the fibres thus causes the yield point to decrease.

When AMP-PNP is washed out from the fibres tension rises isometrically (G—I) and about 60% of the isometric relaxation induced by addition of AMP-PNP (C—E) is restored. Traces I—K and K—L then indicate that this new tension level (I) in rigor has become a new yield point. Washing out AMP-PNP from the fibre thus causes the yield point to increase again.

Figure 2 shows the effect of varying the AMP-PNP concentration (up to 5 mM) on isometric tension, the yield point, immediate elastic modulus and static elastic modulus. It is seen from Figure 2 that the yield point (●) of the fibre is practically equal to the isometric tension level (○) characteristic for the respective AMP-PNP concentration used. Figure 2 shows further that immediate elastic modulus (□) re-

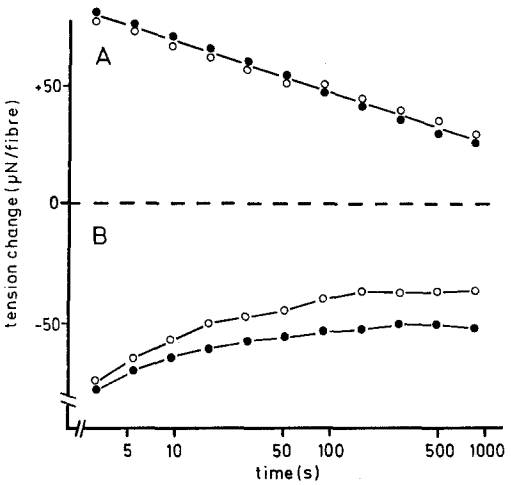

Fig. 3. Time course of tension adjustment [(●), rigor; (○) 0.1 mM AMP-PNP] on a log scale after stretches (**A**) to tension higher than the yield point and releases (**B**) to tensions below the yield point. Amplitudes of length changes 0.3% L_i (L_i = 5.05 mm). Dotted line = tension T_0 before the length change (152 μN/fibre in rigor and 105 μN/fibre at AMP-PNP = 0.1 mM). See also legend to Figure 1

mains nearly the same as that in rigor at an AMP-PNP concentration below 0.5 mM, but that immediate elastic modulus decreases at higher AMP-PNP concentration (1 and 5 mM). On the other hand, static elastic modulus (▽) mainly decreases with increasing AMP-PNP concentration even below 0.1 mM.

Figure 3 shows the time course of tension adjustment towards the steady state level after a length change when the initial tension is equal to the yield point. A stretch (0.3% ΔL_i, *A*, Fig. 3) produces a slow tension transient. During this transient tension values decrease linearly with increasing logarithm of time in rigor (●) and in fibres irrigated with AMP-PNP (○). In rigor preparations with and without AMP-PNP no plateau of the tension-time curve could be observed within 1000 s. The time course of the tension change induced by a stretch of the same amplitude (0.3% ΔL_i) did not differ for fibres in rigor (●) and for fibres irrigated with 0.1 mM AMP-PNP (○). In other words the tension transients were the same for preparations relaxed by AMP-PNP and for preparations in rigor. However, it should be noted that the tension level (Fig. 1, *E*) before stretch was lower for the fibres charged with AMP-PNP than for the fibres in rigor state (cf. Fig. 1, *C*). After a release (−0.3% L_i, *B*, Fig. 3) tension recovers towards a plateau level which is higher in AMP-PNP irrigated fibres than in fibres in rigor: the static elastic modulus of AMP-PNP charged fibres is lower than the static elastic modulus of fibres in rigor.

A rigor contraction can also be generated when ATP is removed from a contracting fibre (pCa = 5). In this case, the initial rigor tension is higher (up to 500 μN/fibre) than when rigor is generated by removing ATP from a relaxing solution (normal rigor, cf. Kawai and Brandt). The high rigor tension is not stable: under isometric conditions the high tension slowly decreases. The time course of this tension decay is similar to that observed in normal rigor after stretching the fibre beyond the yield point (cf. Fig. 3). In both cases there is a linear relation between the tension change and the logarithm of time. Moreover, the slope derived from such a plot is practically the same both in the case of the isometric tension level reached after high rigor tension (transfer from contraction to rigor solution) and in the region of plastic flow of normal rigor (Fig. 3) in the case of the stress-strain relaxation.

However, Ca ions do not change isometric tension, immediate stiffness, nor the yield point when EGTA or CaEGTA is added to the fibre in an existing rigor state: in the presence and in the absence of AMP-PNP the mechanical behaviour in high rigor or normal rigor is then not affected at all.

Discussion

Glycerol-extracted fibrillar muscle fibres in rigor exhibit a clearly defined yield point which is manifested in a sudden change of the slope of tension versus length curves (cf. *B–A–C*, Fig. 1) and by the different time courses of tension adjustment after stretch or release near the yield point (cf. Fig. 3). These findings are similar to the results from studies on iodoacetic acid-poisoned frog sartorius muscles (Maréchal, 1960; Lowy and Mulvany, 1973; Mulvany, 1975).

A plastic response to a strain of a fibre may be produced by a slippage of cross bridges which would thereby detach from the actin filament and reattach at an other locus of that filament. Thereby the elastic element within the cross bridge would be released. Addition of AMP-PNP lowers the yield point (cf. *F–E–G*, Fig. 1), while removal of AMP-PNP partially restores the initial yield point of the rigor state (cf. *I–K–L*, Fig. 1), so that addition and subsequent removal of AMP-PNP lowers the initial rigor tension. This constitutes evidence that removal and subsequent addition of AMP-PNP under isometric conditions produce a slippage of cross bridges.

One might argue that plastic flow occurs in structures other than cross bridges (e.g. *Z*-line filaments) and that a property of such structures determines the yield point, however, since the yield point was reversibly affected by AMP-PNP and this compound is known to bind to the bridges, this seems to be unlikely.

When AMP-PNP is washed out from the fibres under isometric conditions tension increases (↑, Fig. 1) at a nearly constant value of immediate stiffness, i.e. with a presumably constant number of cross bridges attached to the actin filament thus confirming earlier results (Beinbrech et al., 1976). This indicates that the elastic elements of the attached cross bridges are restretched upon removal of AMP-PNP under isometric conditions. During the wash out of AMP-PNP from the fibres cross bridges do not slip appreciably, but rather generate tension when they undergo a change in conformation (Huxley, 1969), this presumably from a perpendicular position to an acute angled position (cf. Beinbrech et al., 1976). Thus it is likely that the effect of adding AMP-PNP to fibres is to induce conformational changes of cross bridges attached to the actin filament as well as to give rise to an increased tendency for the cross bridges to slip along the actin filament.

Recent binding studies have shown that AMP-PNP is bound to the active centre of the myosin heads stretch dependently and reversibly (Kuhn, 1977). The fact that static stiffness of AMP-PNP charged fibres is significantly lower than static stiffness in rigor can be interpreted in terms of a stretch dependent AMP-PNP binding: when a stretch increases the amount of AMP-PNP bound to the myosin heads, these additionally charged heads induce a partial relaxation of tension. On the other hand, a release of the fibre induces a decrease of AMP-PNP bound to the myosin heads. These additionally released heads rotate into the acute angled position, thereby generating enhanced recovery of tension. Thus, for stretch and release static elastic modulus is lowered by the length dependent AMP-PNP binding.

At tension below the yield points, however, cross bridges seem to be attached to the actin in a stable manner. If cross bridges were to break and be reformed, tension would approach to an equilibrium value. This adjustment of tension would be observed within the half-life of a bridge. However, in fibres held at initial length L_i as well as in fibres released by 1.2% L_i rigor tension did not change appreciably (after an initial stress-strain relaxation) for prolonged observation times (up to 24 h). Thus, there are force maintaining cross bridges in fibrillar muscle fibres which have an astonishingly long half-life. From statistical mechanical considerations it seems to be rather unlikely that an individual cross bridge has such a long half-life: the binding energy of a single acto-myosin link certainly does not exceed several thousand times the mean energy of temperature movement (kT) which would be necessary to guarantee such long half-lives. It is therefore postulated that force generating cross bridges act cooperatively by aggregating into clusters along the actin filament; the free energy of such clusters is dissipated when slippage of cross bridges takes place along the actin filament. The slow and complex (multi-exponential) time course of the tension transient observed after a stretch to regions where the fibres flow plastically (Fig. 3) gives a further hint that force-maintaining cross bridges are cooperatively aggregated when slippage occurs.

Two different kinds of sarcomere states were observed in idoacetic acid poisoned frog skeletal muscles (Mulvany, 1975): intact and damaged. When these muscle preparations were stretched by 20% L_0, the average length of intact sarcomeres increased by less than 5%. This finding may be taken to indicate that stretches beyond the yield point induce a slippage of *all* cross bridges in some sarcomeres (which are then presumably damaged) whereas other sarcomeres – namely those with tightly bound cross bridges – remain rather unaffected by such stretches.

Since the binding energy of single cross bridges is assumed to be cooperatively stored, the free energy which determines the stability of a cluster may become much greater than the mean energy of temperature movement. In consequence, the clustered cross bridges become stable when the number of cross bridges within it becomes large. (For energetics of phase transitions in linear systems, see e.g. the Ising model, cf. Birshtein and Ptitsyn, 1966.)

It would then be interesting to know whether, when AMP-PNP lowers the yield point, it weakens the interaction energy between the myosin heads and the actin sites, or whether adding AMP-PNP causes the number of cross bridges within a cluster to decrease.

Under elastic conditions of the fibre (tension lower than the yield point) the force maintaining cross bridges have half-lives greater than 24 h for the breaking of actomyosin linkages. But, even in the absence of plastic deformations, static stiffness in rigor was found to be only about 60% of immediate stiffness in rigor. This indicates that stress-strain relaxation processes occur over a period of 20 min in rigor. [A quick phase, i.e. a rapid tension adjustment within 10 ms, was not observed (cf. Heinl et al.).] Among other possibilities [swelling of sarcomeres (Rome, 1972), viscosity within the cross bridges (Abbott and Steiger, 1977)] one could suggest an attractive hypothesis for the interpretation of these stress-strain relaxations as follows: even in the absence of slippage of cross bridges due to plastic flow there exist two distinct cross bridge populations: firstly the cross bridges which are aggregated into clusters and which are able to maintain tension, secondly cross bridges which attach

to the actin filament and detach from the actin. These latter processes would be equilibrium processes and the cross bridges of this second population would not contribute to steady state tension. The attachment and detachment of the *non* force-maintaining cross bridges would be altered by a lengthening via a change of the actual concentration of the detached myosin heads at a fixed locus on the actin filament. Since all cross bridges which are attached to the actin at the moment of the length change contribute to the tension increase induced by the lengthening, one would expect a tension decrease after stretching the fibre resulting from the cross bridge population tending to an equilibrium. Experiments to check this hypothesis by measuring the elastic modulus *during* very fast length changes (duration 0.2—0.4 ms) are in progress.

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