Journal of Physiology (1989), **410**, *pp.* 351–366
With 4 text-figures
Printed in Great Britain

DISSOCIATION OF FORCE FROM MYOFIBRILLAR MgATPase AND STIFFNESS AT SHORT SARCOMERE LENGTHS IN RAT AND TOAD SKELETAL MUSCLE

BY D. GEORGE STEPHENSON, ANGUS W. STEWART* AND GREGORY J. WILSON

From the Department of Zoology, La Trobe University, Bundoora, Melbourne, Victoria 3083, Australia

(*Received 30 June 1988*)

SUMMARY

1. Single fast-twitch fibres from the extensor digitorum longus muscle of the rat, *Rattus norvegicus*, and single twitch fibres from the iliofibularis muscle of the cane toad, *Bufo marinus*, were mechanically skinned and then used to measure maximally Ca^{2+}-activated ($[Ca^{2+}] > 0{\cdot}03$ mmol l^{-1}) isometric force production, myofibrillar MgATPase activity and fibre stiffness at different sarcomere lengths. MgATP hydrolysis was linked by an enzyme cascade to the oxidation of NADH (nicotinamide adenine dinucleotide, reduced form) and was monitored by a microfluorimetric system. Fibre stiffness was measured from the amplitude of force oscillations generated by small sinusoidal length changes.

2. At sarcomere lengths which were optimal for isometric force production (around 2·7 μm for rat and 2·2 μm for toad fibres) the myofibrillar MgATPase activity (mean $\pm$ S.E.M.) at 21–22 °C was found to be $3{\cdot}80 \pm 0{\cdot}53$ molecules MgATP hydrolysed s^{-1} per myosin head for eight rat fibres and $6{\cdot}35 \pm 0{\cdot}77$ s^{-1} per myosin head for four toad fibres.

3. At sarcomere lengths shorter than 2·7 μm in rat fibres and 2·2 μm in toad fibres, MgATPase and stiffness remained elevated and close to their respective values at 2·7 μm in rat fibres and 2·2 μm in toad fibres even when the isometric force decreased to near zero levels.

4. The dissociation at short sarcomere lengths of myofibrillar MgATPase activity and fibre stiffness from isometric force suggests that the cross-bridge cycle is not greatly affected by double actin filament overlap with the myosin filaments at short sarcomere lengths. Moreover, the results suggest that cross-bridges can be formed by myosin with actin filaments projecting from the nearest Z-line and from the Z-line in the other half of the sarcomere.

5. These results help to reconcile energetic and mechanical data obtained by others at short sarcomere lengths and can be explained within the framework of the sliding filament theory.

* Present address: Department of Science, Western Australian College of Advanced Education, Doubleview, Perth, Western Australia 6018, Australia.

INTRODUCTION

Muscle contraction involves the formation of actomyosin cross-bridges which utilize chemical energy liberated by the hydrolysis of MgATP to develop force between the filaments (Huxley, 1969; Huxley, 1974; Woledge, Curtin & Homsher, 1985). Careful investigation of the isometric force–sarcomere length relation (Ramsey & Street, 1940; Gordon, Huxley & Julian, 1966*a*, *b*) has provided insight into the mechanism of force generation in striated muscle. A great deal more could be learned about the molecular mechanism of muscle contraction if one could unequivocally answer the question whether the myofibrillar MgATPase activity is always coupled in the same fashion to isometric force generation.

Earlier studies by Aubert (1956) have indicated high levels of maintenance heat during isometric tetanic contraction of frog muscle at short sarcomere lengths. In view of the later results of Gordon *et al.* (1966*b*) these observations could have suggested that the myofibrillar MgATPase becomes partially uncoupled from force generation at short sarcomere lengths. However, results obtained with tetanically stimulated frog muscle at different sarcomere lengths by Infante, Klaupiks & Davies (1964), who measured inorganic phosphate production, and Sandberg & Carlson (1966), who measured the hydrolysis of creatine phosphate, suggested that the myofibrillar ATPase, like isometric force, declined abruptly at short sarcomere lengths. This conclusion was further supported by the observations of Hayashi & Tonomura (1968) with bundles of glycerinated mammalian fibres in which these investigators measured the MgATPase activity at different sarcomere lengths.

A careful examination of the biochemical data from a current point of view reveals that the experiments of Hayashi & Tonomura (1968) were conducted in solutions with no added Ca^{2+} or Ca^{2+} buffers and therefore the fibres were most probably not maximally activated at short sarcomere lengths where the sensitivity of the contractile apparatus for Ca^{2+} is known to decrease (Stephenson & Wendt, 1984). Also, the tetanized frog muscles of Infante *et al.* (1964) and Sandberg & Carlson (1966) may not have been fully activated at short sarcomere lengths (Taylor & Rudel, 1970; Rudel & Taylor, 1971).

In view of the importance of knowing whether isometric force becomes uncoupled from myofibrillar MgATPase at short sarcomere lengths, we decided to re-investigate the relationship between isometric force and myofibrillar MgATPase with mechanically skinned fibre preparations which can be activated under controlled ionic conditions at different sarcomere lengths (Ashley & Moisescu, 1977; Moisescu & Thieleczek, 1978; Stephenson & Williams, 1981, 1982). In the present study we have used not only anuran fibres on which most mechanical and energetic experiments were done in the past (Woledge *et al.* 1985) but also mammalian fast-twitch fibres which are better suited for work on the ascending limb of the force–sarcomere length diagram because of their significantly longer actin filaments (ter Keurs, Luff & Luff, 1981). In addition, we have measured relative stiffness in the fibres at different sarcomere lengths as another indicator of cross-bridge formation (Ford, Huxley & Simmons, 1981).

Some of the results presented here were communicated to meetings of the Aus-

tralian Physiological and Pharmacological Society (Stewart, Wilson & Stephenson, 1988; Wilson, Stephenson & Stewart, 1988).

METHODS

Isolation and preparation of fibres

Adult cane toads (*Bufo marinus*) were killed by double pithing of the central nervous system. Adult rats (*Rattus norvegicus*, Long–Evans Hooded strain) were killed by deep anaesthesia with diethyl ether. The iliofibularis muscles of the cane toad and the extensor digitorum longus (EDL) muscles of the rat were ablated and kept until used at 5 °C in paraffin oil (Ajax) which was saturated with water. Twitch (cane toad) and fast-twitch (rat) fibres were dissected and mechanically skinned under the paraffin oil as previously described (Stephenson & Williams, 1981, 1983; Fink, Stephenson & Williams, 1986).

Simultaneous measurement of isometric force and myofibrillar MgATPase activity

The skinned fibres were mounted with surgical silk (Deknatel 10) between two stainless-steel hooks, one of which was on the arm of a micro-manipulator and the other of which was attached to a sensitive piezoresistive force transducer (AME-802) as described previously (Stephenson & Williams, 1981). After dissection, all preparations were transferred for at least 10 min to a relaxing solution containing EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N,N'*- tetraacetic acid) (50 mmol l^{-1}, $[Ca^{2+}] < 1$ nmol l^{-1}) and Triton X-100 (2% v/v) to disrupt the intracellular membraneous compartments (Stephenson, Wendt & Forrest, 1981; Fink *et al.* 1986) in order to eliminate ATP-dependent membrane pumps like the Na^+–K^+ pump and the sarcoplasmic reticulum Ca^{2+} pump. The hydrolysis of MgATP to ADP and inorganic phosphate (P_i) by actomyosin (AM) was coupled via an enzyme cascade to the oxidation of NADH (nicotinamide adenine dinucleotide, reduced form) to NAD^+ using pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Takashi & Putnam, 1979; Griffiths, Guth, Kuhn & Ruegg, 1980; Guth & Junge, 1982) according to the following reaction scheme:

$$\text{ATP} \xrightarrow{\text{AM}} \text{ADP} + \text{P}_i$$

$$\text{PEP} + \text{ADP} \xleftarrow{\text{PK}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xleftarrow{\text{LDH}} \text{lactate} + \text{NAD}^+.$$

All solutions contained (mmol l^{-1}): phosphoenol pyruvate (PEP), 12; NADH, 10; total ATP, 8; Mg^{2+}, 1; $EGTA^{2-}$ + $CaEGTA^{2-}$ + $HDTA^{2-}$ (hexamethylenediamine-*N,N,N',N'*-tetraacetic acid), 50; K^+, 141; Na^+, 16; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) pH buffer, 60 (pH 7·10±0·01); P^1, P^5-di-(adenosine-5')-pentaphosphate, 0·1; PK, 104 units ml^{-1}; LDH, 168 units ml^{-1}. All experiments were performed at 21–22 °C. High concentrations of PK, LDH and PEP were used to ensure that the lag between fluorescence changes and ATP hydrolysis was negligibly small during the MgATPase measurements (Guth & Wojciechowski, 1986). P^1, P^5-di-(adenosine-5')-pentaphosphate was used to inhibit adenylate kinases (Lienhard & Secemski, 1973).

The full Ca^{2+} activation of the skinned fibres was achieved by rapidly raising the $[Ca^{2+}]$ within the preparations to about 0·03 mmol l^{-1} using the $[Ca^{2+}]$-jump technique (Moisescu, 1973; Ashley & Moisescu, 1973; Moisescu, 1976; Stephenson & Williams, 1981). After maximum force was reached, the fibres were rapidly transferred to paraffin oil, where the MgATPase activity could be measured with high resolution in a defined length of the fibre segment. The progress of reaction was measured fluorimetrically via an inverted microscope equipped for epifluorescence (Leitz–Diavert) which was focused on a short segment (about 300 μm) in the middle of the muscle preparation (Fig. 1). The fibres were irradiated with UV light from a 50 W mercury arc lamp through a band-pass filter (360–380 nm). An EMI bialkali photomultiplier tube attached to the microscope measured the emitted light once the light had passed through a dichroic mirror and a long-pass suppression filter with a cut-off of 430 nm (Leitz filter block A). Fluorescence and force signals were displayed on a dual-channel chart recorder. The protocol in these experiments involved first incubating the fibre in a relaxing solution with EGTA (50 mmol l^{-1}, $[Ca^{2+}] < 1$ nmol l^{-1}) and then equilibrating

it for 3 min in a relaxing solution with $HDTA^{2-}$ (49·9 mmol l^{-1}) and $EGTA^{2-}$ (0·1 mmol l^{-1}, $[Ca^{2+}] < 10$ nmol l^{-1}). From this relaxing solution the preparation was transferred to paraffin oil in a miniature chamber which also contained the Ca^{2+}-activating solution (Fig. 1). The aqueous and oil components within the chamber remained separated at all times. In order to make the MgATPase measurement the chamber was moved laterally so that the fibre moved through the oil–solution interface into the Ca^{2+}-activating solution. Then, after maximum force was reached, the chamber was moved so that the fibre was transferred back to oil without passing through air and without altering its position in relation to the objective. The fibre was always under oil in the

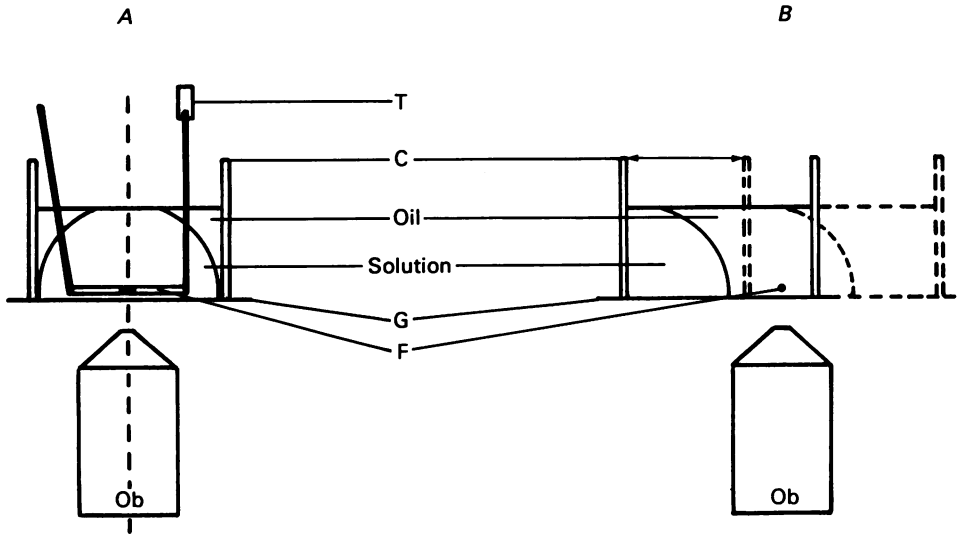

Fig. 1. Diagram showing how fibres were Ca^{2+}-activated during simultaneous measurements of MgATPase activity and isometric force. Paraffin oil and Ca^{2+}-activating solution remain in separate areas of a small circular chamber (C, diameter 15 mm) with a thin glass floor (G, thickness 100 μm). The dark area in the centre of the fibre (F) in *A* shows the part of the fibre upon which the objective (Ob) was focused. T is the force transducer. In *B* is shown a cross-section along the vertical dashed line in *A*. The arrows indicate that the chamber was moved back and forth perpendicularly to the long axis of the fibre so that the whole length of the fibre touched the oil–solution interface at the same time. The fibre and objective remained stationary. The dashed lines in *B* represent the position of the chamber and Ca^{2+}-activating solution while the fibre was in solution.

relaxed state when the focus was adjusted. With this technique we could avoid any evaporative losses from the fibre during activation and we could begin to measure the MgATPase activity from the moment the fibre was transferred to oil. The small amount of NADH carried in the fibre when transferred to oil ensured a very high sensitivity for our measurements (Fig. 2). The total amount of NADH in the field at the fibre was estimated to be around 0·2 nmol per centimetre fibre length for a fibre of 50 μm diameter in oil. The preparation was relaxed soon after all of the NADH had been utilized. The sarcomere length in these experiments was determined by microphotography or by direct measurement with a graticule of the average sarcomere spacings in the preparation in oil, both when the preparation was relaxed and when it was maximally Ca^{2+}-activated.

Simultaneous measurement of isometric force and relative fibre stiffness

Relative fibre stiffness was determined from the amplitude of the force oscillations generated by small sinusoidal length oscillations (2 kHz) of fixed amplitude (< 4 nm/half-sarcomere) applied at one end of the fibres and by comparing the amplitude of the force oscillations with those obtained

at sarcomere lengths at which optimal force production occurred. The fibres were mounted with braided surgical silk between a stainless-steel hook, which was fixed to a force transducer (AME-802) with a resonant frequency of 3·45 kHz and the stainless-steel beam of a vibrating motor (Ling Dynamic Systems, V201, UK) which was driven by an LDS Power Oscillator (TP025, UK). The amplitude of the force oscillations was measured by an absolute value converter, built in the departmental electronics workshop, after having passed through a low-pass filter with a cut-off above 10 kHz. This signal was monitored simultaneously with isometric force on a dual-channel chart recorder (Fig. 3). The use of multifilament surgical silk to attach the skinned fibres to the force-recording apparatus minimized damage to the ends of the fibres compared with other methods of attachment (Moss, 1979). This was checked in a previous study of skinned fibres subjected to the slack-test technique by comparing the intercepts on the length axis at time zero (Julian, Rome, Stephenson & Striz, 1986).

The measurements were made at 21–22 °C in solutions normally used in this laboratory (Stephenson & Williams, 1981, 1983; Fink *et al.* 1986) which had a similar cationic composition to those used in the measurement of MgATPase activity but contained creatine phosphate (10 mmol l^{-1}) and creatine phosphokinase (15 units ml^{-1}) instead of the ATP-regenerating system based on phosphoenol pyruvate. The fibres were fully Ca^{2+}-activated ($[Ca^{2+}] > 0{\cdot}03$ mmol l^{-1}) after pre-equilibration for 2 min in a solution containing $HDTA^{2-}$ (49·9 mmol l^{-1}) and $EGTA^{2-}$ (0·1 mmol l^{-1}, $[Ca^{2+}] < 10$ nmol l^{-1}). The sarcomere length of the fibres in the stiffness experiments was determined using laser diffraction (Stephenson & Williams, 1982; Stephenson & Wendt, 1984). Relative fibre stiffness was near zero for sarcomere lengths shorter than about 2·30 μm in cane toad fibres and 2·80 μm in rat fibres. At longer sarcomere lengths Ca^{2+}-activated fibre stiffness was obtained by subtracting the corresponding value of resting fibre stiffness in relaxing solution from that in maximally Ca^{2+}-activating solution.

RESULTS

MgATPase activity and isometric force

The results shown in Fig. 2 were obtained with one mechanically skinned toad muscle fibre after the membraneous cellular systems were disrupted by exposure to Triton X-100 (2% v/v, see Methods) for 12 min. In Fig. 2*A* the fibre was relaxed and the very slow decrease in NADH fluorescence indicates that there was a very low level of MgATPase in the preparation in the relaxed state. In Fig. 2*B* the same preparation is shown maximally activated by Ca^{2+} at a sarcomere length near optimal for force production and in Fig. 2*C* at short sarcomere length producing very little force.

As the drop in fluorescence was due to the oxidation of NADH, which was directly coupled (coupling factor 1:1) to the hydrolysis of MgATP, the slope of the fluorescence trace gives a direct measure of the amount of MgATP hydrolysed per unit time. Note that the drop in fluorescence is linear until almost all of the NADH in the segment is oxidized. The result obtained at short sarcomere length as shown by the slope of the fluorescence trace in Fig. 2*C* was 2·4 times higher than that at near-optimal filament overlap (Fig. 2*B*). This observation clearly shows that the hydrolysis of MgATP does not decline in proportion to Ca^{2+}-activated isometric force at short sarcomere lengths.

Most, if not all, ATP hydrolysis observed in our experiments is due to actomyosin interaction since at long sarcomere lengths where little overlap between the actin and myosin filaments is expected, the MgATPase rate at full Ca^{2+} activation compared with that at optimal sarcomere length was only about 10% in the rat (three observations from two fibres, Fig. 4*A*) and 16% in the toad (one observation). The

MgATPase results were therefore treated as myosin-related MgATPase. The amount of myosin in the length viewed by the microfluorimetric system at fixed aperture should be proportional to the average sarcomere length, which in the Ca^{2+}-activated fibre in Fig. 2*B* was 2·36 μm and in Fig. 2*C* was estimated to lie between 1·0 and

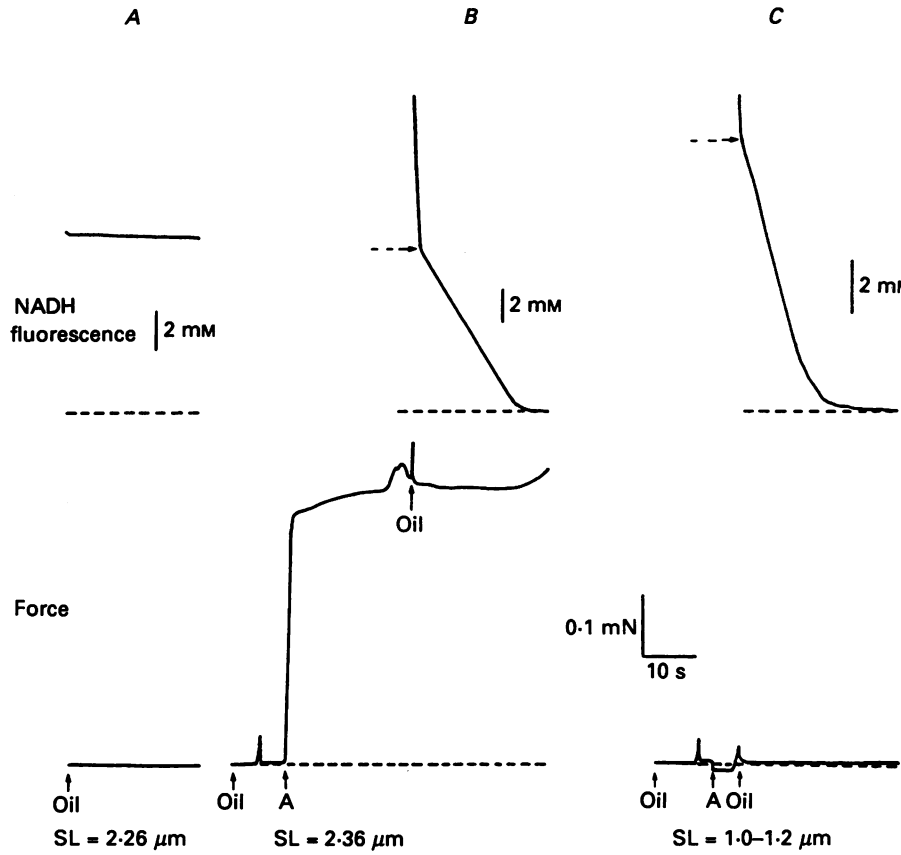

Fig. 2. Simultaneous measurement of MgATPase activity from the slope of the NADH fluorescence traces (upper panels) and isometric force traces (lower panels) in a skinned fibre from the iliofibularis muscle of the cane toad. The fibre was relaxed in *A* and fully Ca^{2+}-activated in *B* and *C*. The estimation of sarcomere length in *C* was made from the irregular sarcomere pattern characteristic of Ca^{2+}-activated fibres at these short sarcomere lengths and from the change in the overall length of the segment. The length and diameter of the fibres in solution at 2·26 μm sarcomere length were 3·5 mm and 65 μm respectively. The dashed lines represent the baselines for the appropriate conditions. Note that the baseline for zero active force in paraffin oil was slightly higher than in aqueous solution (A).

1·2 μm. Thus the amount of myosin in the length of the segment corresponding to Fig. 2*C* was estimated to be between 1·97 (2·36/1·2 μm) and 2·36 (2·36 /1·0 μm) times higher than that in Fig. 2*B*. Considering that the relative amount of MgATP hydrolysed per unit time was about 2·4 times higher in Fig. 2*C* than in Fig. 2*B* and that the myosin content was also higher by a similar factor in Fig. 2*C*, then there was

very little difference in the actomyosin MgATPase activity (per myosin content) at the two sarcomere lengths.

MgATPase activity can be expressed in molecules of ATP hydrolysed per second per myosin head. The difference between the fluorescence levels detected when the fibre was transferred to oil and when all NADH in the segment viewed was oxidized is proportional to the initial NADH concentration in the fibre segment. Since the NADH molecule is not electrically charged, the NADH concentration in the preparation must be very close to the concentration in the bathing solution (Stephenson *et al.* 1981). Therefore one can measure the MgATPase activity in mmol ATP l^{-1} s^{-1} from fluorescence traces such as those in Fig. 2 by equating the difference between the initial and final fluorescence level to the oxidation of 10 mmol l^{-1} NADH and by implication to the hydrolysis of 10 mmol l^{-1} ATP. This MgATPase activity can then be divided by the myosin head concentration (C_M) in the segment of the preparation viewed in order to determine the number of ATP molecules hydrolysed per second by each myosin head. C_M in intact muscle (both cane toad and rat) was taken to be 200 μmol l^{-1} (Yates & Greaser, 1983). In skinned fibres at slack length (at sarcomere lengths of about 2·2 μm for toad and 2·7 μm for rat) C_M is expected to decrease by a factor of 1·69, to 118 μmol l^{-1}, due to swelling of the skinned fibre upon transfer from water-saturated paraffin oil to relaxing solution (Stephenson & Williams, 1983; Fink *et al.* 1986). At sarcomere lengths (SLs) different from 2·2 μm for toad and 2·7 μm for rat skinned fibres, C_M was calculated from the expression:

$$C_M = 118\ \mu\text{mol}\ l^{-1} \times \left(\frac{\text{SL at slack length}}{\text{experimental SL}}\right) \times \left(\frac{A \text{ at slack length}}{A \text{ at experimental SL}}\right), \quad (1)$$

where the ratio between the cross-sectional area at slack length (A at slack length) and at the experimental sarcomere length (A at experimental SL) was estimated from the ratio between the fluorescence attributed to NADH at slack length and that at the experimental sarcomere length with a constant field at the fibre in oil. The larger change in fluorescence in Fig. 2*C* compared with Fig. 2*B* is due to the increase in fibre cross-sectional area at short sarcomere length.

Ca^{2+}-activated fibre stiffness and isometric force

In Fig. 3 are representative paired results obtained from one toad fibre at different sarcomere lengths showing simultaneously measured fibre stiffness and isometric force production. These results show that the signals indicating fibre stiffness and force production rise rapidly in response to activation. When the sarcomere length in the relaxed fibre was decreased from 2·19 to 1·81 μm (Fig. 3*B*) Ca^{2+}-activated force declined to 70% of that obtained with optimal filament overlap at 2·19 μm (Fig. 3*A*). At a sarcomere length of less than 1·4 μm (Fig. 3*C*) Ca^{2+}-activated isometric force production declined to 21%. The level of fibre stiffness, however, was very similar in all three cases.

Comparisons between MgATPase activity, fibre stiffness and isometric force

Isometric force, actomyosin MgATPase activity and fibre stiffness results were obtained at full Ca^{2+} activation at different sarcomere lengths with ten cane toad twitch fibres (Table 1) and twenty-one rat fast-twitch fibres (Fig. 4). At sarcomere

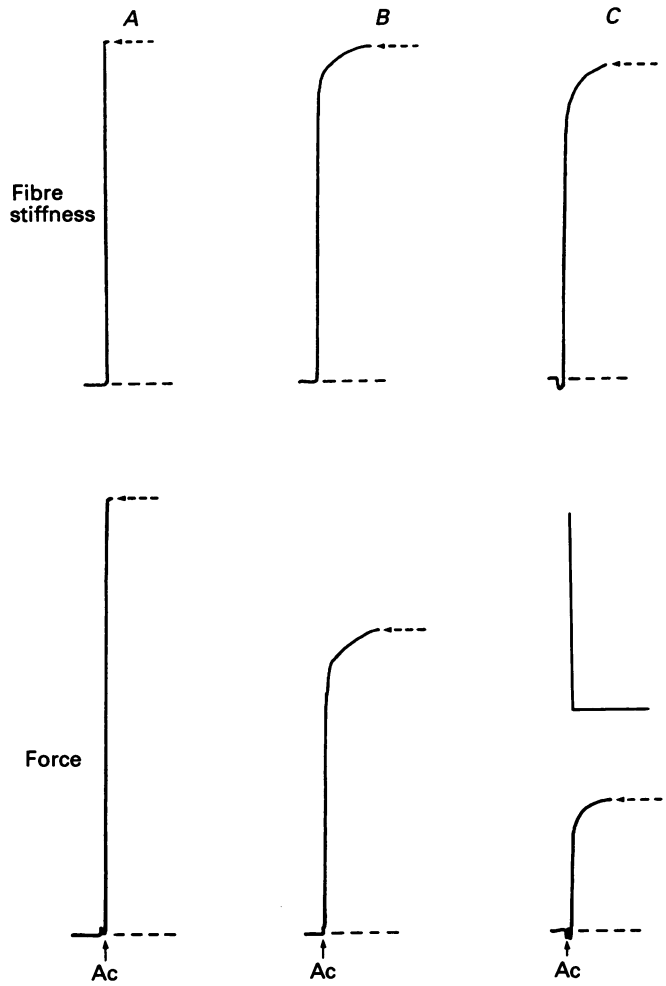

Fig. 3. Representative results showing simultaneously recorded fibre stiffness traces (upper panels) and isometric force traces (lower panels) at various sarcomere lengths in a skinned twitch fibre from the iliofibularis muscle of the cane toad. The fibre was maximally Ca^{2+}-activated (Ac) at different sarcomere lengths. The resting sarcomere lengths in *A* and *B* were 2·19 and 1·81 μm respectively. The final sarcomere length in *C* was $< 1{\cdot}40$ μm. The fibre length at 2·19 μm sarcomere length was 3·4 mm. The diameter measured in oil immediately after skinning was 46 μm and it expanded to 60 μm in solution. The vertical calibration bar represents 0·1 mN for force and 0·02 mN, measuring peak-to-peak amplitude of the force oscillations, for fibre stiffness. The amplitude of the sinusoidal length oscillations was constant at less than 4 nm/half-sarcomere. The horizontal calibration bar represents 60 s. The dashed lines indicate zero stiffness and zero force respectively. The horizontal arrows indicate the maximum levels of stiffness and force reached.

lengths which were optimal for force production in cane toad fibres ($2{\cdot}18 \pm 0{\cdot}02$ μm) MgATPase activity was found to be $6{\cdot}35 \pm 0{\cdot}77$ (mean $\pm$ S.E.M.) s^{-1} per myosin head (four fibres). At sarcomere lengths which were optimal for force production in rat fibres ($2{\cdot}72 \pm 0{\cdot}02$ μm) MgATPase activity for the same experimental conditions was $3{\cdot}82 \pm 0{\cdot}47$ (mean $\pm$ S.E.M.) s^{-1} per myosin head (nine fibres), which is significantly

lower than that for the toad fibres (t test, $P < 0.05$). The MgATPase activity in the relaxed toad fibres was very low (Fig. 2*A*) and was estimated to be 0.06 ± 0.04 s^{-1} per myosin head (mean $\pm$ S.E.M.) from six observations with four cane toad fibres at 2·20–2·26 μm sarcomere length. In the rat EDL the basal rate of MgATPase activity in the relaxed fibres at optimal sarcomere length for force production was 0.15 ± 0.04 s^{-1} per myosin head (ten observations with five fibres). These values indicate that the intrinsic myofibrillar MgATPase activity in the relaxed skinned skeletal fibre preparations is below 1 and 3·9% of the maximum Ca^{2+}-activated MgATPase activity in toad and rat fibres respectively.

TABLE 1. Isometric force, fibre stiffness and myofibrillar MgATPase activity in maximally Ca^{2+}-activated cane toad muscle fibres at different sarcomere lengths

Sarcomere lengths (μm)	Relative force	MgATPase activity (s^{-1} per myosin head)	Relative stiffness
2.18 ± 0.02 (10)	1·00 (10)	6.35 ± 0.77 (4)	1·00 (6)
1.72 ± 0.03 (8)	0.60 ± 0.07 (8)	7.83 ± 1.32 (3)	0.97 ± 0.04 (5)
< 1·40 (6)	0.29 ± 0.10 (6)	5.27 ± 0.50 (2)	1.32 ± 0.28 (4)

Results are given as mean $\pm$ S.E.M. with the number of preparations used in parentheses. Relative force measurements from MgATPase and stiffness experiments are pooled. Below 1·4 μm the sarcomere length could not be accurately measured in skinned fibres from the cane toad. Force and stiffness measurements were normalized to those at 2.18 ± 0.02 μm. See text for explanation of calculation of MgATPase activity. The resting stiffness was near zero in these experiments.

We applied a one-way analysis of variance (ANOVA) assuming the null hypothesis between the means of the maximally Ca^{2+}-activated MgATPase activity data for the cane toad fibres from Table 1. The calculated variance ratio was smaller than the critical value of F even at a 0·10 level of significance. Therefore there is no evidence to conclude that the mean MgATPase activity values obtained at sarcomere lengths of 2.18 ± 0.02, 1.72 ± 0.03 and < 1·4 μm are significantly different from each other. Paired observations with two toad preparations showed that the relative MgATPase activity at sarcomere lengths < 1·4 μm was 0·93 (range 0·60–1·26) of that at sarcomere lengths between 2·20 and 2·26 μm. At 1.72 ± 0.03 μm sarcomere length, paired observations with three toad preparations showed a value of 1.32 ± 0.45 (mean $\pm$ S.E.M.) for relative MgATPase activity compared with that at sarcomere lengths between 2·20 and 2·26 μm. In Table 1 are also shown the values for fibre stiffness at sarcomere lengths of 1.72 ± 0.03 μm and < 1·40 μm compared with that of 2.18 ± 0.02 μm in toad fibres. There was no significant difference in stiffness between the three sarcomere lengths (ANOVA, $P > 0.05$).

In Fig. 4 are shown all of the results for MgATPase activity, relative fibre stiffness and isometric force obtained with maximally Ca^{2+}-activated rat EDL fibres. The results are qualitatively similar to the cane toad observations indicating that both stiffness and MgATPase activity remain elevated as the sarcomere length is decreased below about 2·7 μm, while isometric force decreased markedly to approach zero levels at sarcomere lengths below 1·4 μm. At long sarcomere lengths (around 4 μm) Ca^{2+}-activated isometric force, stiffness and MgATPase all declined in proportion.

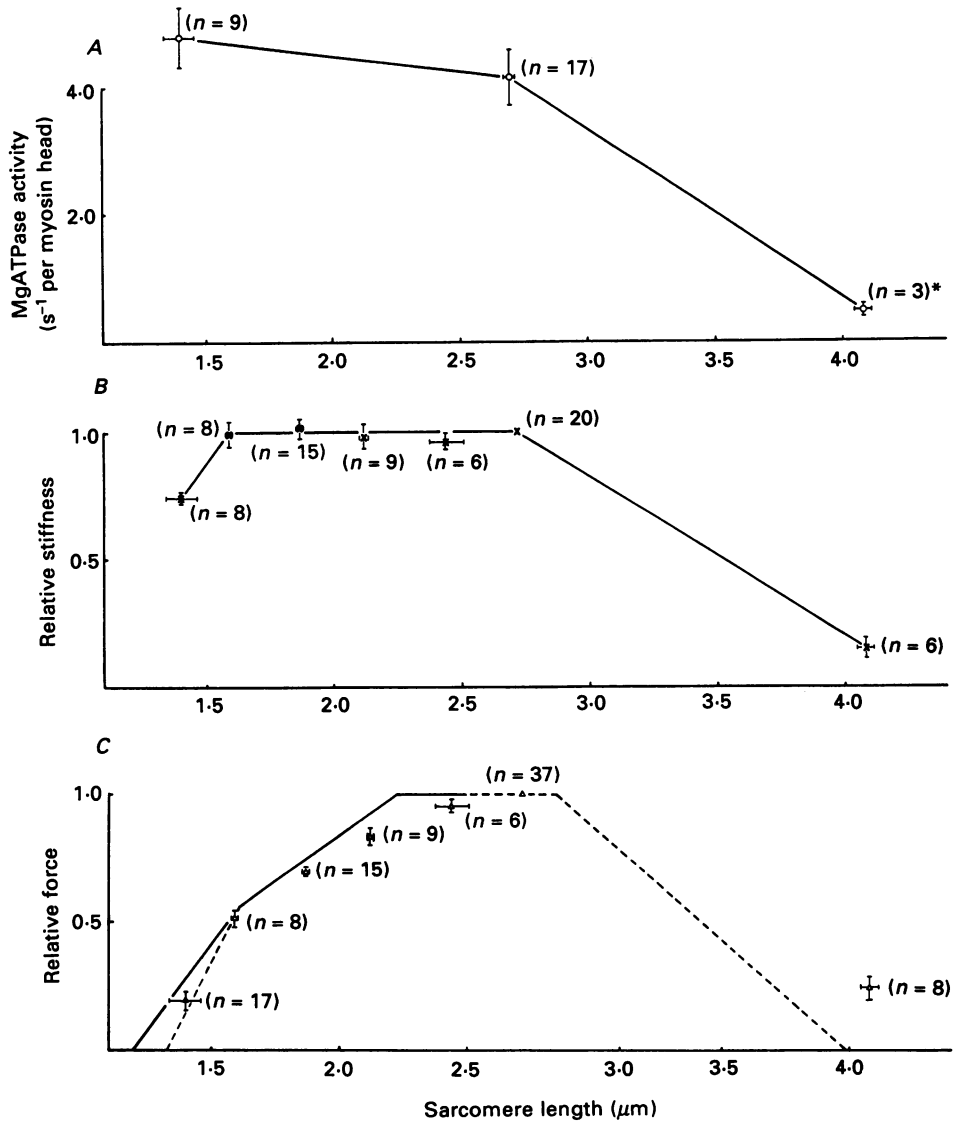

Fig. 4. Sarcomere length dependence of MgATPase (*A*), relative stiffness (*B*) and relative isometric force (*C*) of maximally Ca^{2+}-activated rat EDL skinned fibres. The bars represent the magnitude of the standard errors about the means. The numbers of observations are in parentheses. Lines in *A* and *B* were drawn by eye. The continuous line for relative isometric force (*C*) was obtained assuming that the cycling cross-bridges from the zone of double actin filament overlap do not contribute to net force (see text). The dashed line at sarcomere lengths longer than 2·4 μm is the dependence of isometric force upon sarcomere length in skinned rat EDL fibres, as determined previously in this laboratory (Stephenson & Williams, 1982). The dashed line on the ascending limb was obtained by assuming that a compressive force component exists (see text). The results were obtained using nine fibres in *A* and twelve fibres in *B*. The isometric force data from these twenty-one fibres were pooled in *C*. For sarcomere lengths shorter than 2·8 μm the resting stiffness was near zero. The Ca^{2+}-activated isometric force and stiffness at sarcomere lengths greater than 4 μm were measured above their resting levels. The resting levels for isometric force and stiffness amounted respectively to $0{\cdot}29 \pm 0{\cdot}04$ and $0{\cdot}08 \pm 0{\cdot}03$ (mean $\pm$ S.E.M., $n = 4$ fibres) relative to the maximum Ca^{2+}-activated isometric force and stiffness measured at optimal sarcomere length. *With one of these observations isometric force was not measured.

DISCUSSION

General aspects

A simple technique has been developed based on the coupling of MgATP hydrolysis to NADH oxidation for measuring the MgATPase activity with high resolution in short segments of known sarcomeric pattern along freshly dissected skinned fibre preparations. With this technique it was possible to show clearly that the myofibrillar MgATPase during isometric contractions changed little in maximally Ca^{2+}-activated fibres when the sarcomere length was reduced from optimal values for isometric force production to where very little isometric force could be generated (Figs 2 and 4). The finding that MgATPase activity is dissociated from isometric force at short sarcomere lengths is supported by stiffness measurements. These results strongly suggest that the number and the kinetics of cross-bridges are less affected by double overlap between actin filaments in the zone of cross-bridge formation than is the magnitude of fully Ca^{2+}-activated isometric force.

The dissociation between MgATPase activity and isometric force at short sarcomere lengths offers the most convincing explanation so far for the high levels of maintenance heat measured by Aubert (1956) in tetanized anuran muscle at short sarcomere lengths (Woledge *et al.* 1985). The absolute rates of myofibrillar MgATP hydrolysis obtained in this study with skinned fibres from the rat EDL (3·82 s^{-1} per myosin head) are comparable with other reported values obtained with fast-twitch mammalian muscle fibres under similar experimental conditions. For example Webb, Hibberd, Goldman & Trentham (1986) and Kawai, Guth, Winnikes, Haist & Ruegg (1987) have measured ATPase rates of 3·2 and 3·0 s^{-1} per myosin head respectively in maximally Ca^{2+}-activated single rabbit psoas fibres at 20 °C and 200 mmol l^{-1} ionic strength. In their calculations, both groups assumed a myosin head concentration in their chemically skinned fibres of 200 μmol l^{-1} compared with our estimated value of 118 μmol l^{-1} for mechanically skinned fibres. If we had used the figure of 200 μmol l^{-1} in our calculations, then the maximum MgATPase activity in rat EDL fibres would have been estimated at 2·25 s^{-1} per myosin head for similar conditions. There are not many recent ATPase measurements on anuran fibres at 20–25 °C, but our value of 6·35 s^{-1} per myosin head compares favourably with the ATPase values reported by other groups (Kushmerick & Davies, 1969; Curtin, Gilbert, Kretzchmar & Wilkie, 1974; Homsher, Irving & Wallner, 1981) for intact frog fibres at 0 °C assuming a Q_{10} value of about 2. With a myosin head concentration of 200 μmol l^{-1}, their quoted ATPase values would correspond to about 12, 7·6 and 4 s^{-1} per myosin head respectively at 20 °C. It is interesting that the cane toad fibres showed a 50% higher isometric myofibrillar MgATPase activity than mammalian fibres under identical conditions. This observation is in line with previous studies which showed a consistently higher myofibrillar MgATPase in twitch anuran fibres than in fast-twitch mammalian fibres as discussed by Takashi & Putnam (1979).

Fibre stiffness measurements with toad fibres at sarcomere lengths of 2·18 and 1·72 μm (Table 1) agree very closely with measurements made by Julian & Morgan (1981) on tetanically stimulated intact single frog fibres, which showed that stiffness at a sarcomere length of 1·68 μm was 98% of the stiffness at 2·18 μm. Bressler & Clinch (1975) also reported a tendency of dissociation between isometric force and

stiffness down to a sarcomere length of about 1·5 μm in tetanically stimulated sartorius muscle of the toad (*Bufo bufo*). The large error in our stiffness results from toad fibres at sarcomere lengths below 1·4 μm is most likely to be due to structures other than cross-bridges, like the myofilaments themselves and the Z-lines, interfering with cross-bridge stiffness in a complex way. The stiffness results with rat EDL skinned fibres showed a relatively low scatter (Fig. 4*B*) and demonstrate clearly that there is very little change in stiffness as the sarcomere length decreases from 2·70 μm to where the myosin filaments begin to collide with the Z-lines at 1·61 μm. In a recent study, Allen & Moss (1987) also demonstrated an increase in the ratio between stiffness and tension in maximally activated skinned mammalian fibres at sarcomere lengths shorter than 1·9 μm.

The dissociation between MgATPase activity and isometric force at short sarcomere lengths is contrary to other early observations of whole tetanized muscles (Infante *et al.* 1964; Sandberg & Carlson, 1966) and glycerinated fibres (Hayashi & Tononura, 1968) which suggested that myofibrillar MgATPase activity decreased at short sarcomere lengths similarly to isometric force. There are several reasons to explain the apparent discrepancy between our results and these earlier observations. First, with whole tetanized muscle (Infante *et al.* 1964; Sandberg & Carlson, 1966) it is difficult to distinguish myofibrillar MgATPase from overall ATPase activity and intact muscle may not be fully Ca^{2+} activated at short sarcomere lengths (Taylor & Rudel, 1970; Rudel & Taylor, 1971). Second, as already mentioned, the sarcomere length experiments with glycerinated fibres (Hayashi & Tonomura, 1968) were conducted in solutions with no added Ca^{2+} or Ca^{2+} buffer and the preparations were therefore probably not maximally Ca^{2+}-activated at short sarcomere lengths (Stephenson & Wendt, 1984). Third, the low concentration of MgATP used by Hayashi & Tonomura (1968) could have limited the intramyofibrillar MgATPase activity, particularly at short sarcomere lengths when the diameter of the fibres was larger. Indeed, Hayashi & Tonomura (1968) report in a footnote on p. 110 of their paper that when the ATP concentrations exceeded 2·35 mmol l^{-1} the ATPase activity at very short sarcomere lengths was nearly equal to the maximum ATPase activity.

There were other important differences between these earlier experiments and our experiments which could also be responsible for the different conclusions reached in these studies. For example the glycerinated preparations used by Hayashi & Tonomura (1968) were much larger in diameter than were ours, they developed considerably less tension than could freshly skinned muscle preparations, they had the Ca^{2+}-regulatory system markedly damaged by prolonged glyceration (see also Nakayama, Yamaguchi, Watanabe & Sekine, 1983) and they were activated for long periods of time.

Explanation of results within the framework of the sliding filament theory of muscle contraction

In the classical view of muscle contraction it is postulated that the 'right' polarity of actin and myosin filaments is critical for cross-bridge formation (Huxley, 1969). If this were true, then one would expect that cross-bridges in the region of double actin overlap should only be formed with actin filaments projecting from the nearest

Z-line. Within the general framework of the sliding filament theory of muscle contraction this could further imply that myofibrillar MgATPase, cross-bridge stiffness and isometric force should undergo little change as the sarcomere length decreases from the point where the actin filaments begin to overlap, to the point where the myosin filaments start to be compressed (1·61 μm). Our results clearly show that while this prediction is correct for MgATPase and stiffness measurements, it does not hold for isometric force. However, all of these observations can be simply explained within the general framework of the sliding filament theory if the relative polarity of the actin and myosin filaments to each other is not critical for cross-bridge formation and if there is no net force production as a result of normally cycling cross-bridges in the area of double actin filament overlap. The active component of the isometric force on the ascending limb of the force–sarcomere length diagram would then be determined by the number of cross-bridges which can be formed only in the zone of single overlap of actin filaments with myosin filaments. Cancellation of force in the zone of double actin overlap could arise for example from the action of opposing cross-bridges acting on the same actin filament or if the two heads of a myosin molecule were attached to actins of different polarity in the zone of double actin filament overlap.

The continuous line for relative isometric force in Fig. 4*C* was obtained by assuming that the cycling cross-bridges from the zone of double actin overlap do not make a net contribution to force production. This is to say that fully Ca^{2+}-activated isometric force in relative units (P_r) on the ascending limb of the force–sarcomere length diagram above 1·61 μm, corresponding to the point where the myosin filament (1·56 μm; Page & Huxley, 1963; Craig & Offer, 1976; Julian, Moss & Sollins, 1978) touches the Z-lines (about 0·05 μm; Julian *et al.* 1978), is determined by the relative number of cross-bridges which can be formed only in the zone of single overlap between actin and myosin filaments, i.e.

$$P_r = \frac{\text{myosin filament length} + \text{SL} - \text{actin filament length}}{\text{myosin filament length} - \text{bare region}}. \quad (2)$$

The bare region in these calculations was taken at 0·15 μm (Page & Huxley, 1963; Craig & Offer, 1976) and the actin filament length from end to end through the Z-line was taken as 2·38 μm (ter Keurs *et al.* 1981). At sarcomere lengths shorter than 1·61 μm the myosin filaments become compressed and the isometric force generated by the cross-bridges in the single actin overlap region is given by the following relation:

$$P_r = \frac{(2\ \text{SL}) - \text{actin filament length}}{\text{SL} - \text{bare region}}. \quad (3)$$

The length of the bare region at sarcomere length below 1·61 μm is assumed to drop in proportion from 0·15 μm with the ratio between the experimental sarcomere length and 1·61 μm, where the myosin filaments first make contact with the Z-lines.

The corner in the isometric force–sarcomere length diagram occurring at about 1·6 μm in anuran (Gordon *et al.* 1966*b*) and mammalian fibres (Fig. 4*C*) is likely to be associated with compression of the myosin filaments causing both resistive force (Gordon *et al.* 1966*b*) and a change in force dependence upon sarcomere length. For

the continuous line in Fig. 4*C* no compressive force component was taken into consideration when calculating the force generated at sarcomere lengths shorter than 1·61 μm. If a compressive force component is assumed, then the zero force intercept will occur at sarcomere lengths longer than 1·19 μm as indicated by the dashed line on the ascending limb (Fig. 4*C*).

From our interpretation, due to the longer actin filaments in mammalian than in anuran skeletal fibres, the relative isometric force must be smaller in mammalian fibres at any given sarcomere length under 2·23 μm (eqns (2) and (3)). This prediction is fully supported by our results for rat EDL fibres (Fig. 4*C*) when compared with those of Gordon *et al.* (1966*b*) for frog fibres. The above considerations also imply that MgATPase activity and cross-bridge stiffness would not change below a sarcomere length of about 2·15 μm in anuran fibres and 2·53 μm in mammalian fibres (length of actin filaments + bare zone on the myosin filament), while isometric force would decline from below the sarcomere length where the actin filaments overlap each other and begin to overlap the part of the myosin filaments bearing cross-bridges in the opposite half of the sarcomere (1·85 μm in anuran fibres and 2·23 μm in mammalian fibres).

Experiments with actin filaments symmetrically detached from both Z-lines (Trombitas & Tigyi-Sebes, 1985; Yanagida, Arata & Oosawa, 1985) show that when activated, the free actin filaments stop moving in the middle of the sarcomere, in a position in which they are symmetrical about the M-line and fully overlap each other. These observations provide strong support for the hypothesis that the cross-bridge action between myosin and the double-overlapped actin filaments does not result in net force production.

Thus our proposed interpretation of the ascending limb of the isometric force–sarcomere length diagram provides a unifying basis for the explanation of energetic, mechanical, biochemical and ultrastructural data on vertebrate skeletal muscle.

We are indebted to Professors Sir Andrew Huxley and F. J. Julian for helpful discussions and suggestions concerning experiments and thank Mrs R. Cafarella for skilful technical assistance, and ARGS and NH&MRC for financial support.

REFERENCES

ALLEN, J. D. & MOSS, R. L. (1987). Factors influencing the ascending limb of the sarcomere length–tension relationship in rabbit skinned muscle fibres. *Journal of Physiology* **390**, 119–136.

ASHLEY, C. C. & MOISESCU, D. G. (1973). Tension changes in isolated bundles of frog and barnacle myofibrils in response to sudden changes in the external free calcium concentration. *Journal of Physiology* **233**, 8–9*P*.

ASHLEY, C. C. & MOISESCU, D. G. (1977). The effect of changing the composition of the bathing solutions upon the isometric tension–pCa relationship in bundles of crustacean myofibrils. *Journal of Physiology* **270**, 627–652.

AUBERT, X. (1956). *Le Couplage Energétique de la Contraction Musculaire*. Brussels: Arscia.

BRESSLER, B. H. & CLINCH, N. F. (1975). Cross bridges as the major source of compliance in contracting skeletal muscle. *Nature* **256**, 221–222.

CRAIG, R. & OFFER, G. (1976). Axial arrangement of cross bridges in thick filaments of vertebrate striated muscle. *Journal of Molecular Biology* **102**, 325–332.

CURTIN, N. A., GILBERT, C., KRETZCHMAR, K. M. & WILKIE, D. R. (1974). The effect of the performance of work on total energy output and metabolism during muscular contraction. *Journal of Physiology* **238**, 455–472.

FINK, R. H. A., STEPHENSON, D. G. & WILLIAMS, D. A. (1986). Potassium and ionic strength effects on the isometric force of skinned twitch muscle fibres of the rat and toad. *Journal of Physiology* **370**, 317–337.

FORD, L. E., HUXLEY, A. F. & SIMMONS, R. M. (1981). The relation between stiffness and filament overlap in stimulated frog muscle fibres. *Journal of Physiology* **311**, 219–249.

GORDON, A. M., HUXLEY, A. F. & JULIAN, F. J. (1966*a*). Tension development in highly stretched vertebrate muscle fibres. *Journal of Physiology* **184**, 143–169.

GORDON, A. M., HUXLEY, A. F. & JULIAN, F. J. (1966*b*). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *Journal of Physiology* **184**, 170–192.

GRIFFITHS, P. J., GUTH, K., KUHN, H. J. & RUEGG, Y. C. (1980). ATPase activity in rapidly activated skinned muscle fibres. *Pflügers Archiv* **387**, 167–173.

GUTH, K. & JUNGE, J. (1982). Low Ca^{2+} impedes cross-bridge detachment in chemically skinned *Taenia coli*. *Nature* **300**, 775–776.

GUTH, K. & WOJCIECHOWSKI, R. (1986). Perfusion cuvette for the simultaneous measurement of mechanical, optical and energetic parameters of skinned muscle fibres. *Pflügers Archiv* **407**, 552–557.

HAYASHI, Y. & TONOMURA, Y. (1968). Dependence of activity of myofibrillar ATPase on sarcomere length and calcium ion concentration. *Journal of Biochemistry* **63**, 101–118.

HOMSHER, E., IRVING, M. & WALLNER, A. (1981). High-energy phosphate metabolism and energy liberation associated with rapid shortening in frog skeletal muscle. *Journal of Physiology* **321**, 423–436.

HUXLEY, A. F. (1974). Muscular contraction. *Journal of Physiology* **243**, 1–43.

HUXLEY, H. E. (1969). The mechanism of muscular contraction. *Science* **164**, 1356–1366.

INFANTE, A. A., KLAUPIKS, D. & DAVIES, R. E. (1964). Length, tension and metabolism during short isometric contractions of frog sartorius muscle. *Biochimica et biophysica acta* **88**, 215–217.

JULIAN, F. J. & MORGAN, D. L. (1981). Tension, stiffness, unloaded shortening speed and potentiation of frog muscle fibres at sarcomere lengths below optimum. *Journal of Physiology* **319**, 205–217.

JULIAN, F. J., MOSS, R. L. & SOLLINS, M. R. (1978). The mechanism for vertebrate striated muscle contraction. *Circulation Research* **42**, 2–14.

JULIAN, F. J., ROME, L. C., STEPHENSON, D. G. & STRIZ, S. (1986). The maximum speed of shortening in living and skinned frog muscle fibres. *Journal of Physiology* **370**, 181–199.

KAWAI, M., GUTH, K., WINNIKES, K., HAIST, C. & RUEGG, Y. C. (1987). The effect of inorganic phosphate on the ATP-hydrolysis rate and the tension transients in chemically skinned rabbit psoas fibres. *Pflügers Archiv* **408**, 1–9.

KUSHMERICK, M. J. & DAVIES, R. E. (1969). The chemical energetics of muscle contraction. II. The chemistry, efficiency and power of maximally working sartorius muscles. *Proceedings of the Royal Society* B **174**, 315–353.

LIENHARD, G. E. & SECEMSKI, I. I. (1973). P^1, P^5-di-(adenosine-5′)pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *Journal of Biological Chemistry* **248**, 1121–1123.

MOISESCU, D. G. (1973). The intracellular control and action of calcium in striated muscle and the forces responsible for the stability of the filament lattice. Ph.D. Thesis, University of Bristol, UK.

MOISESCU, D. G. (1976). Kinetics of reaction in Ca-activated skinned muscle fibres. *Nature* **262**, 610–613.

MOISESCU, D. G. & THIELECZEK, R. (1978). Calcium and strontium concentration changes within skinned muscle preparations following a change in the external bathing solution. *Journal of Physiology* **275**, 241–262.

MOSS, R. L. (1979). Sarcomere length–tension relations in frog skinned muscle fibres during calcium activation at short lengths. *Journal of Physiology* **292**, 177–192.

NAKAYAMA, Y., YAMAGUCHI, M., WATANABE, K. & SEKINE, T. (1983). Loss of Ca^{2+}-dependent regulation in glycerinated skeletal muscle contraction. *Japanese Journal of Physiology* **33**, 559–566.

PAGE, S. G. & HUXLEY, H. E. (1963). Filament lengths in striated muscle. *Journal of Cell Biology* **19**, 369–390.

RAMSEY, R. W. & STREET, S. F. (1940). The isometric tension–length diagram of isolated skeletal muscle fibers of the frog. *Journal of Cellular and Comparative Physiology* **15**, 11–34.

RUDEL, R. & TAYLOR, S. R. (1971). Striated muscle fibers: Facilitation of contraction at short lengths by caffeine. *Science* **172**, 387–388.

SANDBERG, J. A. & CARLSON, F. D. (1966). The length dependence of phosphorylcreatine hydrolysis during an isometric tetanus. *Biochemische Zeitschrift* **345**, 212–231.

STEPHENSON, D. G. & WENDT, I. R. (1984). Length dependence of changes in sarcoplasmic calcium concentration and myofibrillar calcium sensitivity in striated muscle fibres. *Journal of Muscle Research and Cell Motility* **5**, 243–272.

STEPHENSON, D. G., WENDT, I. R. & FORREST, Q. G. (1981). Non-uniform ion distributions and electrical potentials in sarcoplasmic regions of skeletal muscle fibres. *Nature* **289**, 690–692.

STEPHENSON, D. G. & WILLIAMS, D. A. (1981). Calcium-activated force responses in fast- and slow-twitch skinned muscle fibres of the rat at different temperatures. *Journal of Physiology* **317**, 281–302.

STEPHENSON, D. G. & WILLIAMS, D. A. (1982). Effects of sarcomere length on the force–pCa relation in fast- and slow-twitch skinned muscle fibres from the rat. *Journal of Physiology* **333**, 637–653.

STEPHENSON, D. G. & WILLIAMS, D. A. (1983). Slow amphibian muscle fibres become less sensitive to Ca^{2+} with increasing sarcomere length. *Pflügers Archiv* **397**, 248–250.

STEWART, A. W., WILSON, G. J. & STEPHENSON, D. G. (1988). Mechanical and energetic measurements in mammalian skeletal muscle at short sarcomere lengths. *Proceedings of the Australian Physiological and Pharmacological Society* **19**, 124*P*.

TAKASHI, R. & PUTNAM, S. (1979). A fluorimetric method for continuously assaying ATPase: application to small specimens of glycerol-extracted muscle fibers. *Analytical Biochemistry* **92**, 375–382.

TAYLOR, S. R. & RUDEL, R. (1970). Striated muscle fibers: inactivation of contraction induced by shortening. *Science* **167**, 882–884.

TER KEURS, H. E. D. J., LUFF, A. R. & LUFF, S. E. (1981). The relationship of force to sarcomere length and filament lengths of rat extensor digitorum muscle. *Journal of Physiology* **317**, 24*P*.

TROMBITAS, K. & TIGYI-SEBES, A. (1985). How actin filament polarity affects crossbridge force in doubly-overlapped insect muscle. *Journal of Muscle Research and Cell Motility* **6**, 447–459.

WEBB, M. R., HIBBERD, M. G., GOLDMAN, Y. E. & TRENTHAM, D. R. (1986). Oxygen exchange between P_i in the medium and water during ATP hydrolysis mediated by skinned fibres from rabbit skeletal muscle: evidence for P_i binding to a force-generating state. *Journal of Biological Chemistry* **261**, 15557–15564.

WILSON, G. J., STEPHENSON, D. G. & STEWART, A. W. (1988). Ca^{++}-activated isometric force and MgATPase activity at short sarcomere lengths in vertebrate skeletal muscle. *Proceedings of the Australian Physiological and Pharmacological Society* **19**, 75*P*.

WOLEDGE, R. C., CURTIN, N. A. & HOMSHER, E. (1985). *Energetic Aspects of Muscle Contraction.* Monographs of the Physiological Society, No. 41. London: Academic Press.

YANAGIDA, T., ARATA, T. & OOSAWA, F. (1985). Sliding distance of actin filament induced by a myosin crossbridge during one ATP hydrolysis cycle. *Nature* **316**, 366–369.

YATES, L. D. & GREASER, M. L. (1983). Quantitative determination of myosin and actin in rabbit skeletal muscle. *Journal of Molecular Biology* **168**, 123–141.