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STATE-DEPENDENT RADIAL ELASTICITY OF ATTACHED CROSS-BRIDGES IN SINGLE SKINNED FIBRES OF RABBIT PSOAS MUSCLE

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SUMMARY

1. In a single skinned fibre of rabbit psoas muscle, upon attachment of cross-bridges to actin in the presence of ADP or pyrophosphate (PP_i), the separation between the contractile filaments, as determined by equatorial X-ray diffraction, is found to decrease, suggesting that force is generated in the radial direction.

2. The single muscle fibres were subjected to compression by 0–8% of dextran T_{500}. The changes in lattice spacings by dextran compression were compared with changes induced by cross-bridge attachment to actin. Based on this comparison, the magnitude and the direction of the radial force generated by the attached cross-bridges were estimated. The radial cross-bridge force varied with filament separation, and the magnitude of the radial cross-bridge force reached as high as the maximal axial force produced during isometric contraction.

3. One key parameter of the radial elasticity, i.e. the equilibrium spacing where the radial force is zero, was found to depend on the ligand bound to the myosin head. In the presence of ADP, the equilibrium spacing was 36 nm. In the presence of $MgPP_i$ the equilibrium spacing shifted to 35 nm and Ca^{2+} had little effect on the equilibrium spacing.

4. The equilibrium spacing was independent of the fraction of cross-bridges attached to actin. The fraction of cross-bridges attached in rigor was modulated from 100% to close to 0% by adding up to 10 mM of ATPγS in the rigor solution. The lattice spacing remained at 38 nm, the equilibrium spacing for nucleotide-free cross-bridges at $\mu = 170$ mM.

5. Radial force generated by cross-bridges in rigor at large lattice spacings (38 nm $\leqslant d_{10} \leqslant$ 46 nm) appeared to vary linearly with lattice spacing.

6. The titration of ATPγS to fibres in rigor provided a correlation between the radial stiffness of the nucleotide-free cross-bridges and the equatorial intensities. The relation between the equatorial intensity ratio I_{11}/I_{10} and radial stiffness appeared to be approximately linear.

7. The fibres under different conditions showed a wide range of radial stiffness, which was not proportional to the apparent axial stiffness of the fibre. If the apparent axial stiffness is a measure of the fraction of cross-bridges bound to actin, it follows that the radial elastic constant is state dependent; or vice versa.

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8. Differences in equilibrium lattice spacing and in radial elastic constant, most probably reflect differences in the molecular structure of the acto-myosin complex and there is more than one single conformation of the various strongly bound cross-bridge states.

9. Determining equilibrium spacings of the radial elasticity appears to be an effective new approach in detecting structural differences among the attached cross-bridges, since this approach is independent of the fraction of cross-bridges attached, a factor that frequently encumbers the interpretation of structural studies of attached cross-bridge states.

INTRODUCTION

In skinned striated muscle fibres, where the surface membrane is made permeable or removed, the spacing between the contractile filaments decreases in transition from the relaxed state to isometric contraction (Shapiro, Tawada & Podolsky, 1979; Matsubara, Umazume & Yagi, 1985; Brenner & Yu, 1985, 1991), or to the rigor state (Maughan & Godt, 1981; Matsubara, Goldman & Simmons, 1984; Brenner, Yu & Podolsky, 1984; Umazume & Kasuga, 1984), suggesting that force is generated in the radial direction. Experimental evidence supports the idea that the radial force is generated by attached cross-bridges. A compelling piece of evidence supporting this view is that the lattice spacing, d_{10}, of the [1, 0] planes of the filament array at non-overlap sarcomere lengths (no cross-bridge attachment) was not influenced by the physiological states, including relaxing, activating and rigor states (Brenner & Yu, 1991). Furthermore, theoretically it was shown that radial force could accompany force generation in the axial direction (Schoenberg, 1980). Recently, radial cross-bridge force was detected in an isometrically contracting intact frog fibre (Gecchi, Bagni, Griffiths, Ashley & Maeda, 1990).

It was shown that the radial force generated by attached cross-bridges in activated and rigor states varied as a function of filament separation (Matsubara *et al.* 1984, 1985; Brenner & Yu, 1985, 1991). At large separations, the direction of the force is toward the centre of the fibre; its magnitude decreases monotonically with decreasing d_{10} until it reaches zero at the equilibrium spacing. The radial force turns expansive if the lattice separation is decreased further. As a first approximation, we represent the radial force F_r generated by cross-bridges in a fibre as the force generated by an assembly of parallel linear springs:

$$F_r \approx nk(r-r_0), \tag{1}$$

where n is the number of cross-bridges attached to actin, k is the elastic constant, and r_0 is the equilibrium spacing. Contrary to the findings of Matsubara *et al.* (1985), Brenner & Yu (1991) showed that the equilibrium spacing in active contraction was significantly different from that in rigor. They proposed that the equilibrium spacing is a function of the attached state of cross-bridges.

If the equilibrium spacing of the attached cross-bridges, hence the overall radial elastic property, is state dependent, it should, for example, also vary with the ligand attached to the myosin moieties. It is well known that nucleotide analogues simulate various states in the cross-bridge ATP hydrolysis cycle and thus may well modify conformation of the attached cross-bridges. In the present study, we have determined

the radial elastic properties from single, chemically skinned rabbit psoas fibres in the presence of the ligands MgATPγS, $MgPP_i$, MgADP. Our results provide further support to the idea that the equilibrium spacing (r_0) is state dependent. Moreover, the radial stiffness *per cross-bridge* (k) is probably also state dependent. Preliminary results have been presented previously (Xu, Brenner & Yu, 1990).

METHODS

Fibre preparations

Rabbits were anaesthetized by subcutaneous injection of ketamine (Ketaset, Fort Dodge Laboratories Inc., IO, USA) at 10 mg (kg of body weight)$^{-1}$ and exsanguinated via the carotid artery. Single, chemically skinned fibres of rabbit psoas muscle were used throughout this study. Fibres were prepared according to Brenner (Brenner, 1983; for details, see Yu & Brenner, 1989). A single fibre was placed between two Mylar windows of a thermoelectrically cooled chamber. The striation pattern of the fibre was monitored continuously by an inverted microscope through a window at the bottom of the chamber. Sarcomere length was 2·3–2·4 μm, which was measured and monitored by laser light diffraction during the X-ray diffraction measurements. Temperature was 5–7 °C, except for ATPγS experiments in which it was 0·5 °C.

Solutions

The following solutions were used for the experiments. (1) Relaxing solution contained (mM): 1 ATP, 3 $MgCl_2$, 1 EGTA, 10 imidazole, pH 7·0, μ = 20 mM; 150 KCl was added for adjusting to μ = 170 mM. (2) Rigor solution contained (mM): 1 EGTA, 1 EDTA, 10 imidazole, 158 KCl, pH 7·0, μ = 170 mM. Before applying the rigor solution, the fibres were rinsed several times by solutions containing (mM): 5 EGTA, 15 EDTA, 20 imidazole, pH 7·0, μ = 70 mM. (3) ADP solution contained (mM): 1 ADP, 3 $MgCl_2$, 1 EGTA, 10 imidazole, 150 KCl, pH 7·0, μ = 170 mM. To ensure against ATP contamination, control experiments were performed with 0·1 unit ml^{-1} of hexokinase (Sigma Chemical Co.) and 50 mM of glucose (Sigma Chemical Co.), 1 mM dithiothreitol (DTT) and 0·25 mM of the myokinase inhibitor diadenosine pentaphosphate (Ap_5A, Sigma Chemical Co.) added to the ADP solution. (4) PP_i solution contained (mM): 4 PP_i, 6 $MgCl_2$, 10 imidazole, 1 DTT, 0·25 AP_5A, either 1 EGTA or 0·5 CaEGTA, pH 7·0; 135 mM KCl was added to increase the ionic strength to μ = 170 mM. (5) The composition of ATPγS solutions is listed in Table 1.

Commercial ATPγS obtained from Boehringer Mannheim GmbH was purified to remove ADP and other contaminating nucleotides by DEAE-Sephadex A25 chromatography (Th. Krafts & B. Brenner, unpublished method).

Equatorial X-ray diffraction

Lattice spacings were determined by equatorial X-ray diffraction. The lattice spacing, d_{10}, defined for the distance between the Bragg [1, 0] planes, is related to the separation, d, between the thick filaments by $d = d_{10}/(\sin 60 \text{ deg})$. X-ray diffraction patterns were recorded by using a rotating anode X-ray generator (Elliott GX-6) and a single wire position-sensitive X-ray detector (Yu & Brenner, 1989). Integrated intensities, centroids and widths of the peaks of equatorial reflections were calculated by using the software programs supplied by the data acquisition system PCA-I (Nucleus Inc., Oak Ridge, TN, USA). The intensities and the spacings of the Bragg planes [1, 0] and [1, 1] were taken as the mean values of the two sides of the patterns.

Exposure time for each pattern was generally 500 s. To minimize the effect of radiation damage on the fibre, the X-ray exposure point was shifted along the fibre so that the total exposure time at the same spot was no more than 4000 s. In the experiments using pyrophosphate, total exposure time at the same spot was less than 1500 s. The skinned fibre preparation in the presence of PP_i appears to be very susceptible to radiation damage (Brenner, Yu, Greene, Eisenberg & Schoenberg, 1986).

Estimates of radial force and radial stiffness of attached cross-bridges

To estimate the radial force generated by cross-bridges as a function of lattice spacing, we obtained two sets of response curves to applied radial force (by osmotic pressure) from the same fibre

(i.e. d_{10} as a function of applied radial force): (1) the response curve for the fibre with the ligand, and (2) the response curve of the same fibre under relaxing condition. The difference in the radial force applied to the fibre with ligand and to the same fibre under relaxing condition to reach the same lattice spacing is equal to the radial force generated by the attached cross-bridges. The point where the two response curves cross each other signifies the condition where no radial force is generated by the attached cross-bridges. This lattice spacing is defined as the equilibrium spacing (for details, see Brenner & Yu, 1991).

TABLE 1. Composition of solutions containing ATPγS

	ATPγS				
	0 mM	0·1 mM	1·0 mM	2·0 mM	10 mM
Imidazole (mM)	10	10	10	10	10
$MgCl_2$ (mM)	2	2	2	2	2
CaEGTA (mM)	1	1	1	1	1
Glucose (mM)	50	50	50	100	150
KCl (mM)	155	155	150	145	105
DTT (mM)	1	1	1	1	1
Hexokinase (units ml^{-1})	0·01	0·01	0·1	0·2	0·5
AP_5A (mM)	0·25	0·25	0·25	0·25	0·25

pH = 7·0, μ = 170 mM.

The external radial force by osmotic pressure was achieved by adding the anhydroglucose polymer dextran T_{500} (Pharmacia Fine Chemicals Inc., Uppsala, Sweden; weight-averaged molecular weight $MW_w \simeq 470000$; number-averaged molecular weight $MW_n \simeq 170000$). Concentrations of dextran T_{500} ranging between 0 to 8% (w/v) were used in the experiments. The osmotic pressure of the solutions containing T_{500} was measured directly by Brenner & Yu (1991) (see Table 2), using a micro-osmometer (Knauer Co., Wissenschaftkliche Geräte, K. G., Oberursel-Taunus, Germany) containing a cellulose membrane with cut-off at molecular weight 20000 (Schleicher & Schuell, Germany). The osmotic pressure produced by dextran T_{500} used in the present study was assumed to be the same as those listed in Table 2.

From the osmotic pressure exerted by dextran T_{500}, one may calculate the applied radial force, F_r, between the thick and the thin filaments in units of pN (single thick filament)$^{-1}$ with the length of the thick filament assumed to be 1·6 μm. The expression used is $2\sqrt{3}\Pi d_{10}$, where Π is the applied osmotic pressure due to dextran T_{500}; d_{10} is the lattice spacing of the Bragg plane [1, 0] of the filament lattice (Schoenberg, 1980; Matsubara *et al.* 1984; Rau, Lee & Parsegian, 1984).

Radial stiffness at the equilibrium spacing is defined as the slope of the curve of radial force *vs.* lattice spacing at the equilibrium spacing.

Experimental procedures

Diffraction patterns were generally obtained in increasing concentrations of dextran while keeping the basic solutions the same (i.e. either in relaxing solution or ATP analogue-containing solutions). A set of equatorial patterns from fibres under relaxing condition was always paired with a set of patterns obtained from the same fibres in ligand-containing solutions.

The data points, i.e. recorded d_{10} *vs.* radial force applied by dextran T_{500} obtained under each condition, were curve-fitted by MLAB (Knott, 1979) to single exponential functions of the form $(A \exp(bx) + C)$. At each lattice spacing, the radial force generated by attached cross-bridge is calculated by subtracting the fitted curve for the state under study (e.g. ADP) from the corresponding fitted curve for the relaxed condition.

Probable errors in the values of equilibrium spacings and radial stiffness were estimated as the following: curves were first fitted to mean values ± S.E.M. of d_{10} by the same type of equations used for the mean values. Differences were then taken between curves in all four combinations (e.g. the curve fitted for mean values + S.E.M. of the relaxed condition subtracted by the curve for mean

TABLE 2. Osmotic pressure of relaxing solutions (μ = 170 mM) containing dextran T_{500}

Dextran (% (w/v))	Osmotic pressure × 10^2 (Pa)
1	1·85±0·14
2	5·36±1·03
3	11·0±2·00
4	20·3±4·93
5	34·5±7·81
6	58·6±7·56
7	86·2±2·60
8	123·0

Mean values±S.D. of osmotic pressure determined from three batches of dextran T_{500}. The value at 8% dextran T_{500} was obtained by extrapolating the osmotic pressure *vs.* concentration curve by eye.

TABLE 3. Equilibrium spacings and radial stiffness of attached cross-bridges under various conditions

	Equilibrium spacing d_{10} (nm)	Radial stiffness at equilibrium spacing (pN (thick filament)$^{-1}$ nm^{-1})
Normal rigor	37·8±0·2	340±8
MgATPγS		
0 mM	37·7±0·2	302±13
0·1 mM	37·3±0·6	114±5
1 mM	37·3±0·7	32±1
2 mM	37·3±1·8	12±9
MgADP	36·4±0·2	215±6
$MgPP_i$		
$-Ca^{2+}$	34·9±0·6	74±3
$+Ca^{2+}$	35·3±0·6	97±13
Active	33·8±0·9	92±8

The data for active fibres were taken from Brenner & Yu (1991); other values are means±probable error. The probable error for the active radial stiffness was calculated from differences of three out of four possible combinations of fitted curves (see Methods). The fourth difference yielded a radial stiffness of 403 pN (thick filament)$^{-1}$ nm^{-1}. With such a large deviation from the other three values, we consider it highly unlikely. Note that compositions for normal rigor and 0 mM ATPγS solutions are not identical, probably causing the differences in the values of the radial stiffness.

values−S.E.M. of the ADP condition) that resulted in four curves of radial force *vs.* d_{10}. The average deviation from the mean value of the equilibrium spacing is quoted as the probable error. The average deviation of the radial stiffness is similarly determined and defined as probable error.

Other forms of functions, such as two-exponential functions, were also tried, but the differences in the estimates of the radial force and equilibrium spacings were insignificant compared to the errors estimated from the curves fitted to the mean values±S.E.M. of d_{10} (see Table 3).

RESULTS

Effect of the number of attached cross-bridges on equilibrium spacing of the radial elasticity

Response of fibres to osmotic pressure in various concentrations of MgATPγS

To vary the number of attached nucleotide-free cross-bridges we titrated ATPγS into the fibre in rigor. We have shown that ATPγS can be used as an ATP analogue

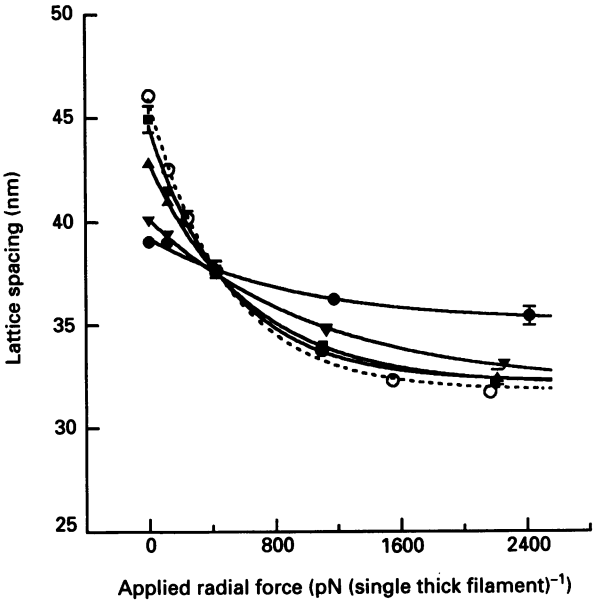

Fig. 1. Response of fibres in various concentrations of MgATPγS to applied radial force. Dextran T_{500} varied between 0 and 8% (w/v); $\mu = 170$ mM; sarcomere length = 2·3–2·4 μm; temperature = 0·5 °C; 24 fibres were used in this study. ○, relaxed fibres; ●, 0 mM ATPγS; ▼, 0·1 mM ATPγS; ▲, 1 mM ATPγS; ■, 2 mM ATPγS. Error bars show ± S.E.M. Dashed line is the curve fitted with a single exponential function to the relaxed data. Continuous lines are curves fitted with single exponential functions to the data in MgATPγS of 0, 0·1, 1 and 2 mM respectively. Data obtained at 10 mM MgATPγS are indistinguishable from those under relaxed condition. The equilibrium spacings are at d_{10} = 37·7, 37·3, 37·3 and 37·3 nm in MgATPγS of 0, 0·1, 1 and 2 mM respectively.

for inducing weak-binding cross-bridges. However, unlike ATP, titration of ATPγS into rigor fibres does not lead to active cross-bridge turnover at micromolar concentration (Kraft, 1990; Kraft, Yu & Brenner, 1990). At $\mu = 170$ mM, cross-bridges with ATPγS are mostly detached from actin, such that one can study radial elasticity arising from different number of attached, nucleotide-free cross-bridges.

To saturate cross-bridges in the fibre with MgATPγS in the presence of Ca^{2+}, it is necessary to use 10 mM MgATPγS and a temperature as low as 1 °C (Kraft, 1990). Therefore concentrations of MgATPγS ranging from 0 to 10 mM and a temperature of 0·5 °C were chosen for this experiment. Dextran T_{500} was applied at 0–8%, corresponding to radial force between 0 and 2400 pN (thick filament)$^{-1}$. The results are shown in Fig. 1. All the curves were obtained by means of fitting the data points recorded at each concentration of ATPγS using functions of one exponential.

In 0 mM MgATPγS, the equilibrium spacing lies at 37·7 ± 0·2 nm. As the concentration of ATPγS increases from 0 to 0·1, 1, 2 mM, the equilibrium spacing remains nearly the same, at d_{10} = 37·7, 37·3, 37·3, 37·3 nm respectively. In 10 mM MgATPγS the response curve is indistinguishable from that under relaxed conditions (with 1 mM ATP).

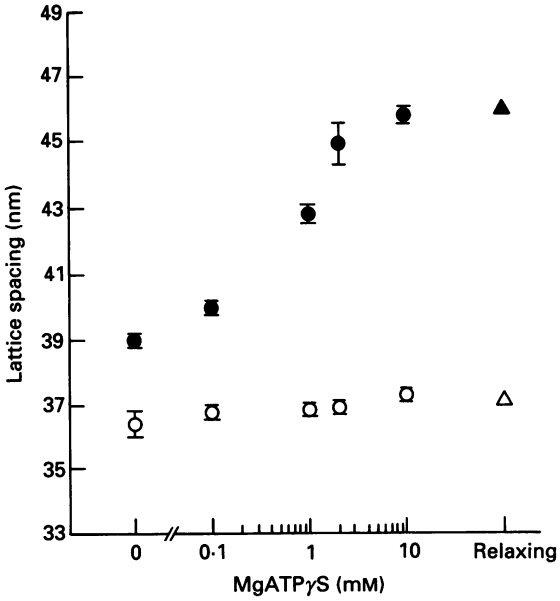

Fig. 2. Evidence showing that number of attached nucleotide-free myosin heads has only very little effect on the lattice spacing in the presence of 4·2% dextran T_{500} when lattice spacing is very near the equilibrium spacing. ○, data from fibres in solutions containing 0–10 mM MgATPγS under 4·2% dextran T_{500}. ●, for comparison, data from fibres in the same solution but with no dextran T_{500} (same data as shown in Fig. 1). Triangles, data from the relaxed fibres with (△) and without (▲) dextran T_{500}. μ = 170 mM. Error bars show ± S.E.M. (n = 5 for both conditions).

Another way of showing that the equilibrium spacing is independent of the number of attached cross-bridges is to pre-compress the fibre to the equilibrium spacing, using 4·2% of dextran T_{500}, and then vary the fraction of rigor cross-bridges by increasing the concentration of ATPγS. Figure 2 shows that the lattice spacing remains nearly the same even though the fraction of attached rigor cross-bridges ranges from ≈ 0 to 100%. In contrast, there is a pronounced change in lattice spacing as a function of ATPγS concentration if osmotic pressure is not applied (Fig. 2). These data points were re-drawn from the starting points (under no osmotic pressure) of the curves in Fig. 1.

Effects of ligand on the equilibrium spacing

Response of the fibres in the presence of MgADP to dextran solutions

Using 1 mM MgADP instead of 1 mM MgATP in an otherwise identical solution under no osmotic pressure, the lattice spacing d_{10} decreased from 45·7 nm (relaxed) to 38·6 nm (Fig. 3). As found for the rigor fibres, the lattice spacing changed

relatively little as dextran T_{500} increased from 0 to 8%. The equilibrium spacing was determined to be 36·4±0·2 nm.

The content of the ADP solution was examined by thin layer chromatography (TLC). No ATP contamination was found. In addition, experiments were carried out

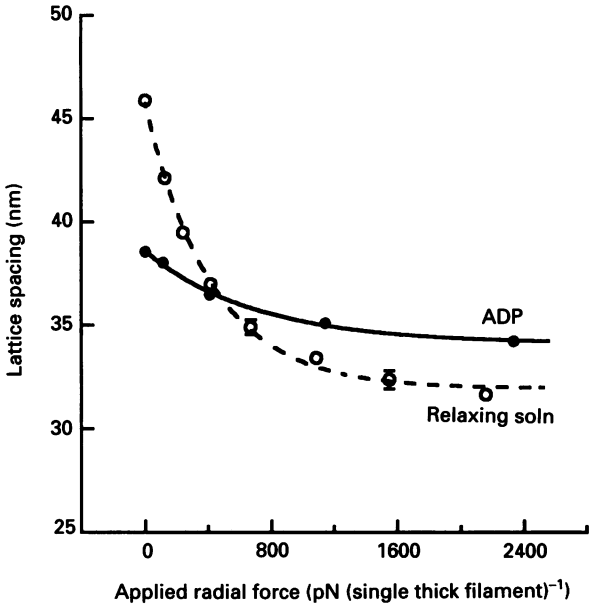

Fig. 3. Responses of fibres to applied radial force in 1 mM MgADP (●) and in relaxing solution (○). Sarcomere length = 2·3–2·4 μm; μ = 170 mM; temperature = 6 °C; 6 fibres were used. Dextran T_{500} varied between 0 and 8% (w/v). Curves are fitted to individual experimental data sets by single exponential functions. The equilibrium spacing is at d_{10} = 36·4 nm.

with solutions containing 1 mM ADP and an ATP-depleting system with hexokinase, glucose and AP_5A (Kraft, 1990; Dantzig, Hibberd, Trentham & Goldman, 1991). The results were indistinguishable from those shown in Fig. 3.

For comparison, a set of response curves to dextran T_{500} of relaxed and rigor fibres was obtained. The results agreed well with previous studies (Brenner & Yu, 1991) in the location of the equilibrium spacing (37·8±0·2 nm) and the magnitude of radial force not under dextran compression at 39·0 nm (290 pN (thick filament)$^{-1}$).

Response of fibres to dextran T_{500} in the presence of 4 mM $MgPP_i$ with and without Ca^{2+}

In the skinned rabbit psoas fibres, large fractions of cross-bridges can attach to actin in the presence of 4 mM $MgPP_i$ at low temperature (6 °C) (Brenner *et al.* 1986). The binding is Ca^{2+} and ionic strength sensitive. At μ = 170 mM, it was estimated from axial stiffness measurements that some 50% of the cross-bridges are attached in the absence of Ca^{2+} and approximately 80% attached in the presence of Ca^{2+} (Brenner *et al.* 1986). Figure 4 shows the response of fibres in 4 mM $MgPP_i$

with and without Ca^{2+} at $\mu = 170$ mM to applied radial force. The response curves in the presence and absence of Ca^{2+} deviate from each other when d_{10} is greater than 36·0 nm. The equilibrium spacing remains the same within experimental errors whether in the absence or in the presence of Ca^{2+} (34·9 ± 0·6 and 35·3 ± 0·6 nm for

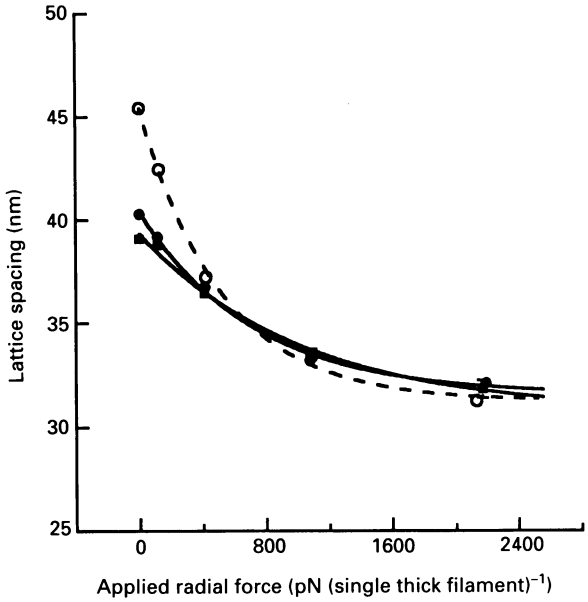

Fig. 4. Responses of fibres to applied radial force in 4 mM $MgPP_i$ with Ca^{2+} (■) and without Ca^{2+} (●), and in relaxing solution (○) at $\mu = 170$ mM; sarcomere length = 2·3–2·4 μm; temperature = 6 °C; 7 fibres were used. Dextran T_{500} varied between 0 and 8% (w/v). Curves are fitted to individual experimental data sets by single exponential functions. The equilibrium spacings are at 35·3 nm for $MgPP_i + Ca^{2+}$ and at 34·9 nm for $MgPP_i + Ca^{2+}$.

$MgPP_i - Ca^{2+}$ and $MgPP_i + Ca^{2+}$ respectively). Thus, the difference in the response curves $\pm Ca^{2+}$ is most probably due to a difference in the fraction of cross-bridges attached to actin.

Estimates of radial stiffness

The radial force generated by attached cross-bridges is clearly a function of filament separation. Figure 5*A* and *B* demonstrates this point more directly for cross-bridges in various concentrations of ATPγS and in rigor, ADP, PP_i. The curves of radial force *vs.* d_{10} were calculcated by subtracting the fitted curve under each condition from the curve of the corresponding relaxing condition in Figs 1, 3 and 4. The radial stiffness at the equilibrium spacing is the slope of each individual curve.

The radial force generated by the attached cross-bridges with bound ADP or PP_i could be very significant. When not under pressure ($d_{10} \approx 39$ nm), the radial force per single thick filament length generated by these strongly bound cross-bridges is of the same magnitude as the maximal isometric force in the axial direction (≈ 300 pN per cross-sectional area of $2/\sqrt{3}d_{10}^2$, between one thick filament and its six nearest neighbouring thin filaments). The radial stiffness varies in a wide range (Table 3).

The radial stiffness of a fibre in rigor is as much as 4 times that of the axial stiffness in the same state. However, the radial stiffness of a fibre in $MgPP_i \pm Ca^{2+}$ is of comparable magnitude to the stiffness in the axial direction.

Radial force generated by cross-bridges in rigor fibres is linear at large lattice spacings (> 39·0 nm)

Figure 5*A* and *B* shows that, in the region where d_{10} is below 39·0 nm, the radial force is approximately linear. Whether at larger separations the radial force is linear was investigated.

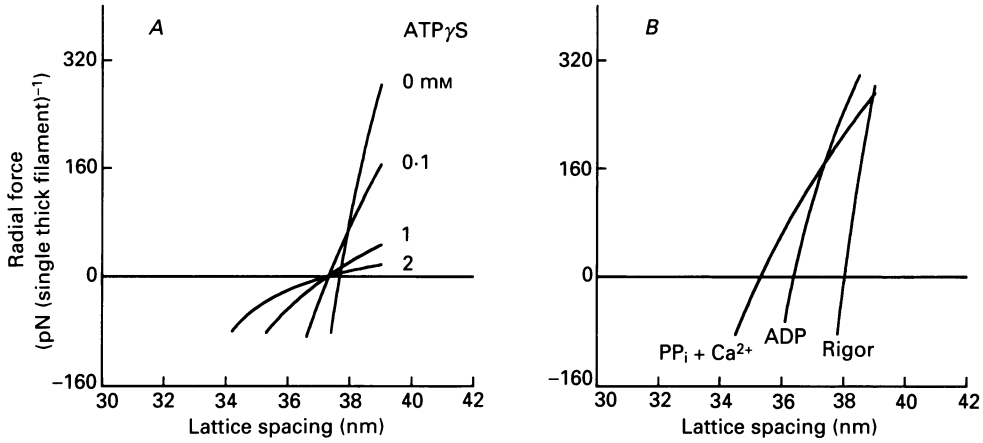

Fig. 5. *A*, radial force generated by cross-bridges in various concentrations of MgATPγS. Each curve was calculated by subtracting the applied radial force for fibres in ATPγS from that for the relaxed fibre at the same lattice spacing. The calculations were obtained by using the fitted curves shown in Fig. 1. *B*, radial force generated by cross-bridges in rigor, in the presence of MgADP, $MgPP_i + Ca^{2+}$ at $\mu = 170$ mM. The radial force was calculated in the same way as in *A*.

At 0 mM of ATPγS (i.e. 100% of the cross-bridges are attached and in rigor), the radial force generated by the rigor cross-bridges and the passive radial force (line *a*, Fig. 6*A*) are balanced at 39·0 nm under no dextran. If we assume that, at lattice spacings greater than 39·0 nm, the attached rigor cross-bridges behave as parallel linear springs with an equilibrium spacing at 37·7 nm (Table 3), the radial force should increase linearly with increasing filament separation (line *b*, Fig. 6*A*). The radial force generated by the muscle fibre is proportional to the fraction of cross-bridges attached, such as those shown as lines *c*, *d* and *e* in Fig. 6*A*. The fraction of nucleotide-free cross-bridges is assumed to be proportional to the radial stiffness at the equilibrium spacing (Table 3). The points where lines *c*, *d* and *e* intersect line *a* are the predicted lattice spacings reached when the fraction of attached rigor cross-bridges is varied. In Fig. 6*B*, the continuous line is the predicted lattice spacing *vs.* fraction of attached rigor cross-bridges with linear elasticity beyond 37·7 nm, and the points are obtained from experiments shown in Fig. 1. The points at 39·0, 40·0, 42·8, 44·9 and 46·0 nm in Fig. 6*B* are found close to the predicted values shown by the

continuous line. Therefore, it appears that the radial force generated by rigor cross-bridges in the region 39·0 nm $< d_{10} <$ 46·0 nm is approximately linear.

Correlation between equatorial intensities I_{10}, I_{11} and the radial stiffness in the presence of ATPγS

Changes in the equatorial intensity ratio I_{11}/I_{10} have been widely used as an indication of mass movement between the thick filament and the thin filament

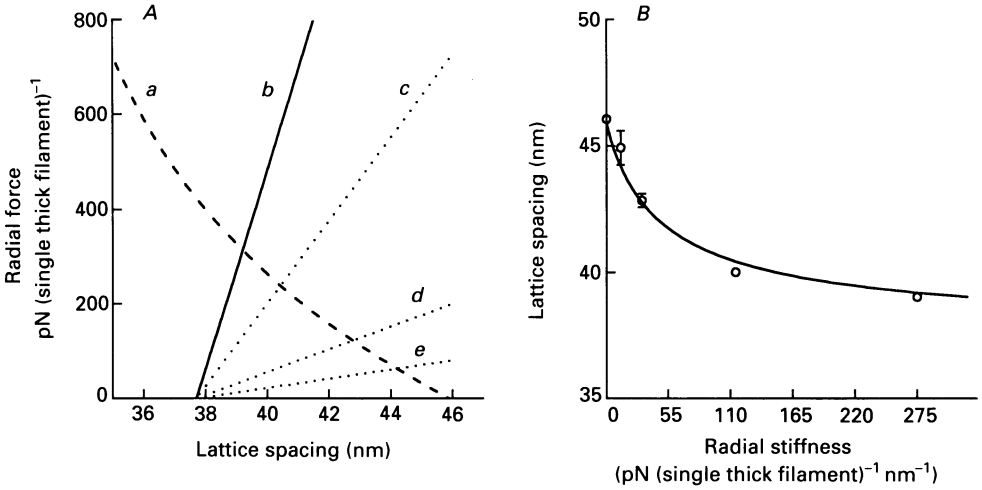

Fig. 6. *A*, changes of the passive radial force and the radial force generated by rigor cross-bridges as a function of lattice spacing d_{10}. Curve *a* is the passive radial force obtained from the fitted curve of relaxed data (Fig. 1, dotted line). Line *b* is the radial force generated by the rigor cross-bridges, approximated as a linear function of d_{10} with equilibrium spacing at 37·7 nm and intersecting curve *a* at 39·0 nm (see text). Lines *c*, *d* and *e* represent radial force as a function of d_{10} generated by 42, 12 and 5% cross-bridges attached respectively, assuming the same equilibrium spacing as rigor and the magnitude of radial force being proportional to fraction of cross-bridges attached in rigor. *B*, continuous line represents calculated d_{10} as a function of increasing radial stiffness of the fibre in the rigor state. The calculation is based on the assumption that radial elasticity of attached cross-bridges is linear at $d_{10} >$ 37·7 nm and the equilibrium spacing is at 37·7 nm (*A*); d_{10} is a result of radial force generated by the cross-bridges being equal to the opposing forces generated by the passive cytoskeletal elements (computed by dashed line in *A*), i.e. when lines *b*, *c*, *d*, etc. intersect curve *a* in *A*. ○, experimental values of d_{10} reached when fractions of rigor cross-bridges are modulated by [ATPγS]. The fractions are estimated from radial stiffness at equilibrium spacing in various concentrations of ATPγS (Table 3).

regions. As the concentration of ATPγS was increased, I_{11}/I_{10} decreased, signifying that the cross-bridges are increasingly detached from the thin filament. At the equilibrium spacing in the ATPγS-containing solutions, while lattice spacing remains constant (Fig. 2), the intensity ratio changed from 2·89±0·34 (mean±S.E.M.) at 0 mM ATPγS to 1·34±0·09 at 2 mM ATPγS. Meanwhile, the radial stiffness changed from 302 to 12 pN (thick filament)$^{-1}$ nm^{-1}. The correlation (Fig. 7) between intensity

ratio and radial stiffness is found to be rather linear. The individual reflections, I_{10}* and I_{11}*, in Fig. 7 are normalized with respect to the I_{10} of the relaxed fibre at μ = 20 mM without dextran. The decrease in I_{10}* and the simultaneous increase in I_{11}* as the radial stiffness increases are monotonic.

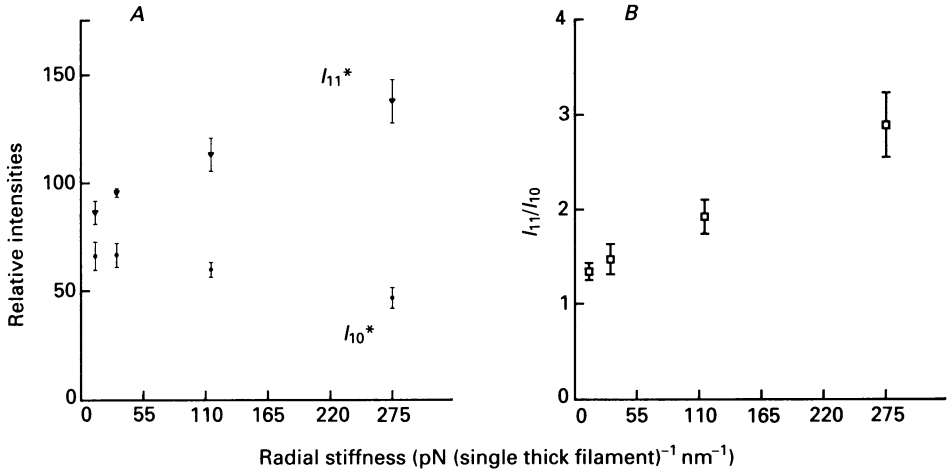

Fig. 7. *A* and *B*, changes in intensities of equatorial reflections, I_{10}*, I_{11}*, and the ratio I_{11}/I_{10}, as a function of radial stiffness of attached cross-bridges. The individual reflections are normalized with respect to the I_{10} of the same fibres in relaxed state at μ = 20 mM without applied dextran T_{500}. Error bars show ± S.E.M. (n = 5).

DISCUSSION

Previously, it was shown that cross-bridges generate radial force when attached to actin. The studies were confined to the active (Shapiro *et al.* 1979; Matsubara *et al.* 1985; Brenner & Yu, 1985, 1991) and rigor fibres (Maughan & Godt, 1981; Matsubara *et al.* 1984; Brenner *et al.* 1984; Umazume & Kasuga, 1984; Brenner & Yu, 1991). In the present study, we further show that attached cross-bridges with a variety of ligands also generate force in the radial direction and it similarly varies as a function of filament separation. That the radial force is generated by attached cross-bridges with various ligands is supported by the result that at non-overlap sarcomere lengths (> 4·0 μm), responses to dextran T_{500} (up to 6% w/v) were not affected by the presence or absence of nucleotides (ATP and ADP) and PP_i (data not shown), similar to the results reported previously (Brenner & Yu, 1991). Evidence for radial force production obtained thus far, however, is limited to the attached cross-bridge states where the binding of myosin to actin is strong (i.e. the strong-binding states; for review, see Brenner, 1990). Evidence for radial force generation by the weak-binding cross-bridges has been inconclusive (Brenner & Yu, 1991).

Some of the elastic properties in the radial direction of the attached, strong-binding cross-bridges are similar: (i) radial force is a relatively linear function of lattice spacing; (ii) at large filament separation the radial force is compressive and it turns expansive once the lattice is compressed below a certain spacing; (iii) this spacing, defined as the equilibrium spacing, is independent of the number of cross-bridges attached.

Equilibrium spacing is independent of the number attached within the same cross-bridge state

At the equilibrium spacing, the radial force generated by attached cross-bridges is zero. If the cross-links formed by the attached cross-bridges between the filaments form parallel elastic systems, the equilibrium spacing (r_0 in eqn (1)) should not be affected by the number (n in eqn (1)) of cross-bridges attached to actin in that state. This concept was supported in the case of activated fibres in a previous study (Brenner & Yu, 1991). In this study, the fraction of nucleotide-free myosin heads was varied. Yet, the equilibrium spacing remained at 37·7 nm. This was shown by two different experiments: (1) by obtaining response curves in several concentrations of ATPγS (Fig. 1); and (2) by pre-compressing the fibres and then titrating ATPγS into the fibre. Since the ionic strength was chosen such that essentially all myosin heads with ATPγS bound will be detached from actin, increasing the concentration of ATPγS would decrease the number of attached nucleotide-free myosin heads. Therefore we conclude that equilibrium spacing is not affected by the number of attached cross-bridges within the same state.

The equilibrium spacing is state dependent

The results reported in this study, combined with our earlier results (Brenner & Yu, 1991), indicate that the position of the equilibrium spacing (r_0 in eqn (1)) is characteristic of each attached cross-bridge state. Even if the probable errors were taken into consideration, the positions of the equilibrium spacing are distinct from each other (Table 3). The lowest value of the equilibrium spacing is found in the Ca^{2+}-activated fibres at 33·8 nm (Brenner & Yu, 1991) while the highest is found in the rigor fibre at 37·8 nm. Intermediate equilibrium spacings are associated with cross-bridges with bound ADP and PP_i as ligands.

It might be argued that the changing of equilibrium spacing with cross-bridge states could reflect a changing mixture of one-headed *vs.* two-headed binding of the cross-bridges to actin. However, this is unlikely in view of the results of ATPγS titration shown in Fig. 1. Electron paramagnetic resonance (EPR) studies of skinned fibres (Fajer, Brunsvold & Thomas, 1988; Pate & Cooke, 1988) indicated that in the presence of nucleotides the two heads of the cross-bridges bind to actin with different affinities. As the concentration of ATPγS increases, the myosin head with the weaker affinity should detach first, followed by the second one. If the equilibrium spacing for cross-bridges with both heads attached is different from that of the single-headed ones, the equilibrium spacing should shift with increasing ATPγS concentration. However, the equilibrium spacing remains almost unchanged (Fig. 1 and Table 3). The small difference (37·7 *vs.* 37·3 nm) found under 0 ATPγS and 0·1–2 mM ATPγS appeared to be insignificant. However, even if it is significant resulting from changing from two-headed nucleotide-free cross-bridge attachment to one-headed attachment, this difference cannot explain the large range of equilibrium spacings found under various conditions studied thus far.

It is also unlikely that the distinct equilibrium spacings are due to differences in charge distributions on the contractile filaments, since the presence of the cations Ca^{2+} and Mg^{2+} does not have a significant effect on the equilibrium spacings as

shown in this (Fig. 4) and previous studies (Brenner & Yu, 1991). The small difference, found for $MgPP_i$ with and without Ca^{2+} (Fig. 4, Table 3), cannot explain the results. On the other hand, binding of nucleotides or their analogues to myosin heads has profound effects on the equilibrium spacing. Therefore, electrostatic charge distribution cannot be the major factor that influences the location of the equilibrium spacing.

Lack of linear correlation between axial stiffness and radial stiffness in the skinned fibre; possibility of stiffness (axial or radial) being state dependent

The present results indicate that radial stiffness of the muscle fibre does not vary in direct proportion with axial stiffness measured previously. The magnitude of measured axial stiffness of a muscle fibre depends on the speed of stretch (for review, see Brenner, 1990). If the stretches are sufficiently fast, the measured stiffness becomes speed independent (reaches a plateau), and its magnitude could be used to determine the fraction of cross-bridges bound to actin. Axial stiffness measured at slower speeds provides an *underestimate* of the fraction bound. In the rabbit psoas muscle fibres, all of the cross-bridges are bound to actin in rigor (Cooke & Franks, 1980; Lovell & Harrington, 1981). In the presence of MgADP, the axial stiffness is nearly the same as that of the muscle in rigor (Schoenberg & Eisenberg, 1987). Yet, the values of the radial stiffness under these two conditions are significantly different from each other ($\approx 1{\cdot}6:1$, Table 3). In a fully Ca^{2+}-activated rabbit fibre under our conditions, the axial stiffness is $\geqslant 85\%$ of the rigor fibre (Brenner, 1991) but the radial stiffness is only $\approx 30\%$ of that of rigor fibre (Brenner & Yu, 1991; Table 3). Furthermore, our earlier mechanical studies indicated that in 4 mM $MgPP_i$ with and without Ca^{2+} at $\mu = 170$ mM and 5 °C, approximately 80 and 50% of the cross-bridges are attached to actin respectively (Brenner *et al.* 1986). Yet their radial stiffness under these conditions is disproportionately less than that of the fibre in rigor and in MgADP (Table 3). Considering that in most cases the axial stiffness was measured at speeds below the plateau region, the disparity between the axial and the radial stiffness is even more striking.

The cause of the disproportionate changes in the axial and the radial stiffness is not known. However, if the axial stiffness is taken as a measure of the fraction of cross-bridges attached to actin, it follows that the radial stiffness *per cross-bridge* (i.e. k in eqn (1)) changes with the state of the attached cross-bridge. On the other hand, the converse, i.e. the axial stiffness is state dependent, could be true instead. For lack of evidence to the contrary, it has been generally assumed that axial stiffness is independent of the state of the attached cross-bridges. The validity of this concept, however, has not been closely examined. A third possibility is that axial stiffness is a measure of the fraction of myosin molecules interacting with actin, regardless of whether the interaction involves one or both heads of the myosin molecule, while the radial stiffness is a measure of the fraction of myosin heads attached. Fajer *et al.* (1988) and Pate & Cooke (1988), based on EPR studies, suggested that in the axial direction, a single-headed nucleotide-free attached cross-bridge was as stiff as a double-headed nucleotide-free one. Finally and quite likely, the overall elastic properties, i.e. in both directions, might be functions of the state of the cross-bridge. Although the present data are insufficient to support any one of the possibilities

proposed above, the present result none the less raises intriguing questions on the concept of state-independent stiffness (axial and radial) for attached cross-bridges.

A new approach in detecting structural differences of attached cross-bridges

The distinct equilibrium spacings most probably reflect differences in the molecular structure of the attached cross-bridges, more precisely the cross-links formed between the thick filament surface and the thin filament by the cross-bridges. For example, flexibility of the myosin head or the attachment mode (the angle of attachment) may be modified by the attached ligand. Although the exact nature of the structural difference is not yet known, these results indicate that there is more than one conformation of the strongly bound actomyosin cross-link other than that of rigor. This conclusion is supported by our recent finding based on equatorial X-ray diffraction that the attached conformation of cross-bridges in fully Ca^{2+}-activated fibres differs from those in the rigor fibres (Brenner & Yu, 1992).

With a significant number of cross-bridge states having been examined, the equilibrium spacing proved to be a sensitive mechanical parameter under a wide variety of conditions. Determining equilibrium spacings represents an effective approach in detecting structural differences of the actomyosin cross-link complex. The significance of this approach lies in the fact that it is independent of the number of cross-bridges attached. This is a distinct advantage over several other techniques for structural studies. For example equatorial X-ray diffraction intensities are known to be affected not only by the conformation of the attached cross-bridges but also by the number of cross-bridges attached to actin (Lymn, 1978; Yu, 1989). Therefore, we believe that determining the equilibrium spacings is a valuable technique as a first-step screening for structural differences in the attached cross-bridges.

Correlation between the intensity ratio I_{11}/I_{10} and radial stiffness

Changes in the intensity ratio I_{11}/I_{10} has been widely used as an indication of cross-bridge formation between the thick and the thin filaments (Huxley, 1968; Haselgrove & Huxley, 1973). An increase in the ratio has been correlated with increasing isometric force level (Yu, Hartt & Podolsky, 1979; Brenner & Yu, 1985). However, as was mentioned earlier, the intensities are affected both by the fraction of cross-bridges attached and by the conformation of attached heads. The correlation between X-ray intensities and active isometric force level may not apply to other conditions. In the present study, as the concentration of ATPγS is increased, the fraction of attached nucleotide-free myosin heads is modulated from 100 to 0%, while the average conformation of the attached heads presumably remains the same. Figure 7 shows that the relation between radial stiffness and I_{11}/I_{10} appears to be linear while somewhat non-linear between radial stiffness and the intensities of the individual reflections. Assuming that the radial stiffness is proportional to the fraction of myosin heads bound to actin, the data provide a correlation hitherto unavailable between the equatorial intensities and the fraction of nucleotide-free acto-myosin crosslinks present in the fibre. This finding should place considerable constraint on models of the nucleotide-free actomyosin structure (Lymn, 1978; Yu, 1989).

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