

Research Paper

Effects of submaximal activation on the determinants of power of chemically skinned rat soleus fibres

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Reducing the activating calcium concentration with skinned fibres is known to decrease isometric force and maximal shortening velocity, both of which will reduce the peak power. However, power is also a function of the curvature of the force–velocity relationship, and there is limited information on how changes in activating calcium affect this important property of muscle fibres. Force–velocity relationships of permeabilized single type I fibres from rat soleus muscle were determined using isotonic contractions at 15°C with both maximal (pCa 4.5) and submaximal activation (pCa 5.6). The rate of tension redevelopment (k_{tr}), which provides a measure of sum of the apparent rate constants for cross-bridge attachment and detachment ($f_{app} + g_{app}$) following a rapid release and restretch, was also measured. Compared with pCa 4.5, specific tension (P_o) at pCa 5.6 declined by $22 \pm 8\%$ (mean \pm s.d.) and the maximal velocity of shortening (V_{max}) fell by $44 \pm 7\%$, but curvature of the force-velocity relationship (a/P_o) rose by $47 \pm 31\%$, indicating a less concave relationship. The value of k_{tr} declined by $23 \pm 7\%$. The change in a/P_o reduced the impact of changes in P_o and V_{max} on peak power by approximately 25%. Fitting the data to Huxley's model of cross-bridge action suggests that lower activating calcium decreased both the rate constant for cross-bridge attachment (f) and that for detachment of negatively strained cross-bridges (g_2). The fact that V_{max} (and thus g_2) changed to a greater extent than k_{tr} ($f_{app} + g_{app}$) is the reason that reduced activation results in a reduction in curvature of the force–velocity relationship.

(Received 16 June 2010; accepted after revision 30 September 2010; first published online 1 October 2010)

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The ability of a muscle to generate power depends on a number of factors, some of which are quantitative properties, such as the length and physiological cross-sectional area of the muscle, while others, such as the specific tension (P_o), maximal velocity of unloaded shortening (V_{max}) and the curvature of the force–velocity relationship (a/P_o), might be classified as qualitative factors. Different muscles vary in these qualitative properties in ways that fit them to the functional demands placed upon them, and it is evident that this variation extends to motor units within a muscle (Burke *et al.* 1971, 1973) and to individual muscle fibres even of the same myosin heavy chain (MHC) composition (Bottinelli *et al.* 1994; Gilliver *et al.* 2009).

When determining the contractile properties of intact muscles or single fibres, care is usually taken to ensure that the preparation is maximally activated but, in life,

muscles are often, if not generally, used in submaximally activated states, and it is therefore of interest to determine how the factors that determine power output are affected by reducing the level of activation. Isometric force is well known to vary in a sigmoidal relationship with activating calcium (Hellam & Podolsky, 1969; Julian, 1971), and it is now well documented that the maximal velocity of shortening is also reduced with low activating calcium (Julian, 1971; Moss, 1986; McDonald, 2000; Morris *et al.* 2003). The rate of recovery of force following a rapid release and restretch (k_{tr}) is also known to be affected by the activating calcium solution, which is thought to reduce the apparent rate of cross-bridge attachment (f_{app}) while that for detachment (g_{app}) is unaffected, so that the sum of the two rate constants ($f_{app} + g_{app}$) decreases (Brenner, 1988). McDonald (2000) tested fast and slow rat muscle fibres and cardiac myocytes. The focus of that study was on

the slowing of V_{\max} at lower levels of $[Ca^{2+}]$, and curvature of the force–velocity relationship was not discussed but, nevertheless, the rise in relative optimal force (P/P_0) at reduced levels of $[Ca^{2+}]$ indicates that a/P_0 increased too, i.e. the force–velocity relationship became less concave. Otherwise, little attention has been paid to the effect that reduced activation has on curvature of the force–velocity relationship.

There is reason to believe that curvature may be affected by the level of activation, because V_{\max} and k_{tr} influence curvature and both are known to be affected by the calcium concentration. In the two-state Huxley (1957) model of cross-bridge action, curvature of the force–velocity relationship is given by $(f + g_1)/g_2$, where f is the rate constant for attachment, g_1 that of detachment where the cross-bridges can develop force and g_2 the rate of detachment of cross-bridges in compression. The sum of the Huxley rate constants ($f + g_1$) is equivalent to $(f_{app} + g_{app})$, which constitutes the rate constant for tension redevelopment after a rapid release and restretch (k_{tr} ; Brenner, 1988). In the Huxley formulation, the maximal velocity of unloaded shortening is proportional to the rate constant g_2 . If, then, both $(f + g_1)$ and g_2 are reduced at low calcium concentrations, curvature of the force–velocity relationship might increase, decrease or be unaffected depending on the extent of changes in k_{tr} and V_{\max} .

The aims of the present study were firstly, to determine the extent of changes in V_{\max} , k_{tr} and curvature when reducing the activating calcium concentration and, secondly, to explore the changes in cross-bridge kinetics that could account for any change, using the Huxley (1957) model.

Methods

Muscle samples

The tissues used in these experiments were obtained from young adult Sprague–Dawley rats killed using approved Schedule 1 methods (cervical dislocation) for other research projects approved by the local animal research ethics committee of the University of Manchester. This is in accord with the generally accepted guideline of reducing animal numbers to a minimum in biomedical research. The soleus muscles were rapidly excised and small fibre bundles prepared, which were immersed in a relaxing solution (see ‘Solutions’ below) containing 50% (v/v) glycerol at 4°C for 24 h, stored at –20°C, and used within 1 month.

Solutions

Relaxing and maximal activation solutions have been described previously (Larsson & Moss, 1993; Degens *et al.* 1998; Gilliver *et al.* 2009). Relaxing solution contained

(in mmol l^{-1}): 4.5 MgATP, 1 free Mg^{2+} , 10 imidazole, 2 EGTA and 100 KCl. Solution pH was adjusted to 7.0 with KOH. Activating solutions were prepared with Ca^{2+} concentrations of either $10^{-4.5}$ M (pCa 4.5; maximal activation solution) or $10^{-5.6}$ M (pCa 5.6; low calcium activating solution); in addition to Ca^{2+} , they contained 5.3 MgATP, 1 free Mg^{2+} , 20 imidazole, 7 EGTA, 19.6 creatine phosphate; pH adjusted to 7.0 with KOH. The ionic strength of both relaxing and activating solutions was adjusted to 180 mM with KCl.

Preparation of single fibres

The procedures for preparing and measuring the single fibres have been described previously (Larsson & Moss, 1993; Degens *et al.* 1998; Gilliver *et al.* 2009). Briefly, small fibre bundles were placed in relaxing solution containing 1% Triton X-100 for 20 min to permeabilize the membranes and sarcoplasmic reticulum. Individual fibres were carefully pulled from the bundles in relaxing solution and mounted in a permeabilized-fibre test system (400, Aurora Scientific Inc., Aurora, Ontario, Canada), tied with nylon thread to fine insect pins attached to the force transducer (403A, Aurora) and motor arm (312C, Aurora). These were mounted over a moveable stainless-steel plate containing a set of machined wells, each with a glass base. The plate, transducer and motor were mounted on an inverted microscope (Olympus IX71, Tokyo, Japan). The image of the fibre was viewed by a video camera and sarcomere length determined via a Fourier transformation of the sarcomere pattern (900A, Aurora). Sarcomere length of the resting fibre was set at $2.6 \mu\text{m}$ at the start of the experiment and checked at regular intervals thereafter. Fibre diameter was measured at three places while suspended in the air, and cross-sectional areas were calculated assuming the fibre to have a circular cross-section (Degens *et al.* 1999; Degens & Larsson, 2007). No correction was made for swelling of the fibres during skinning. Fibre length was measured to the closest 0.01 mm. All experiments were carried out at 15°C.

Contractile properties

Fibres were subjected to isotonic shortening tests as described previously (Gilliver *et al.* 2009). Briefly, fibres were transferred from pCa 7.0 solution, at which the fibre did not develop force, to maximal (pCa 4.5) or submaximal activation solutions (pCa 5.6). When the isometric force had reached a plateau, the fibre was subjected to four sequences of four isotonic shortening steps, essentially as described by Bottinelli *et al.* (1996). In each sequence, the lever arm moved at a speed sufficient to maintain the muscle force at a predetermined percentage of the isometric force. At the end of the sequence, the fibre was stretched back to its original length while still

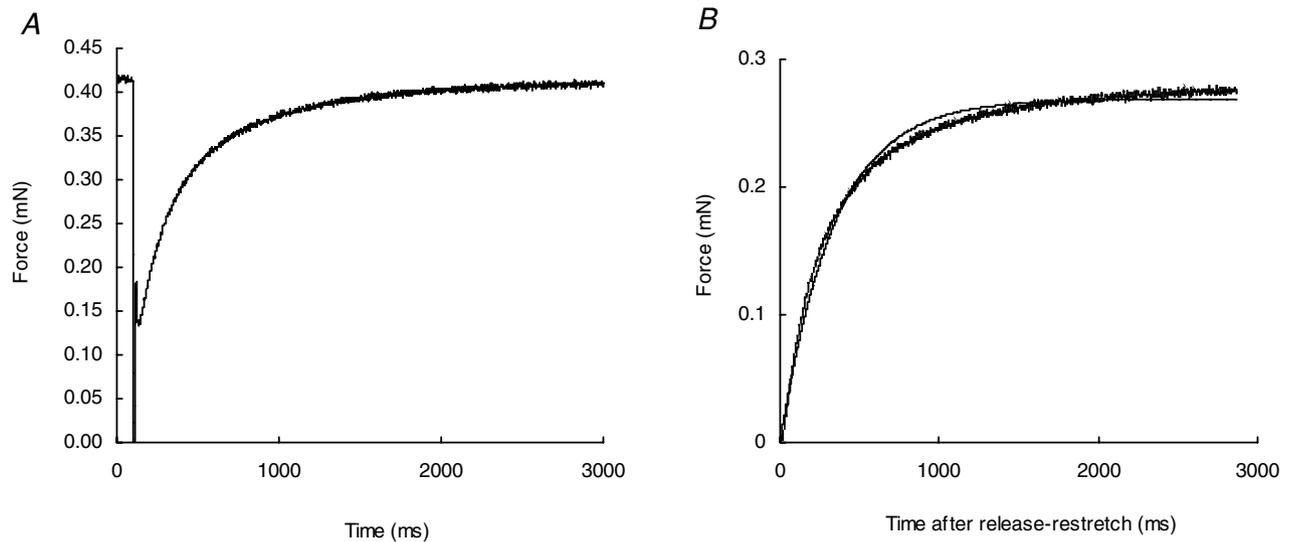


Figure 1. Redevelopment of tension following a rapid release–restretch

A, original force record. B, portion of force trace, starting from point of lowest force following restretch. The data were fitted to a single exponential (continuous line). In this case, the k_{tr} was 2.97 s^{-1} and the fitted line had an r^2 value of 0.99.

in activating solution. This sequence was repeated a total of four times with different percentage values of isometric force.

Subsequently, the rate of force redevelopment (k_{tr}) was determined in varying free calcium concentrations ($[\text{Ca}^{2+}]$). The fibre was moved from pCa 7.0 to pCa 5.6 and then pCa 4.5. In each solution, the fibre was allowed to develop peak force before being rapidly released by 20% fibre length and, after 15 ms, being rapidly restretched to the original length (Fig. 1A).

Data were rejected if the isometric force decreased by more than 10% over the course of the four sequences of isotonic shortening contractions or if it had decreased by more than 10% for the final k_{tr} in pCa 4.5, if the sarcomere length had changed by more than $0.1\ \mu\text{m}$ or if the r^2 values were less than 0.96 when fitting the data to the Hill equation or, in the case of k_{tr} measurements, to the single exponential.

Myosin heavy chain composition of single fibres

After the functional measurements were completed, the fibres were dissolved in Laemmli sample buffer, boiled for 2 min, and the myosin heavy chains separated by SDS-PAGE (Fig. 2), as described previously (Gilliver *et al.* 2009). Only fibres expressing solely the type I MHC were used for further analysis.

Data recording and analysis

Data for force and length were recorded at a sampling frequency of 1 kHz and analysed by fitting least-squares linear regressions to the last 100 ms of the length trace plotted as a function of time, for each step, yielding 16 force and corresponding velocity data points for one fibre (Fig. 3). The force and velocity data from the

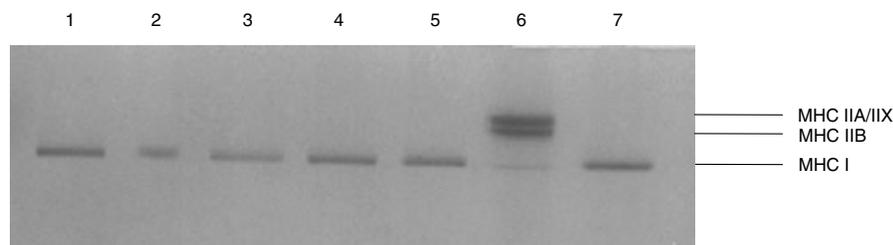


Figure 2. Gel showing type I single muscle fibres in lanes 1–5 and lane 7 alongside a whole muscle extract of rat plantaris muscle in lane 6 to act as band identification

isotonic shortening tests were fitted to the Hill (1938) equation $(P + a) \times (V + b) = (P_o + a) \times b$ using a non-linear least-square regression and an iterative routine written in Matlab (v7.1, The Mathworks Inc., Natick, MA, USA) to give best fit values for the Hill constants a and b , with the force P expressed as a fraction of the isometric force (P_o) recorded just before the start of the isotonic shortening tests. The maximal velocity of unloaded shortening (V_{max}) in fibre lengths per second ($FL\ s^{-1}$) was estimated as $b \times P_o/a$. Maximal power (in watts per litre) was given by $M^2 \times P_o \times V_{max}$, where M is the fraction of either P_o or V_{max} at which maximal power occurs and is derived from a/P_o (Woledge *et al.* 1985, p. 49).

For each k_{tr} test, data from the point at which force started to rise were fitted to the following single exponential: $P = A_o[1 - \exp(-k_{tr}t)]$, where P is force, A_o is the final steady-state force and t is the time, using a non-linear least-squares regression (Solver, Microsoft Excel) giving the apparent rate constant k_{tr} (Fig. 1B).

Data were fitted to the Huxley (1957) model with the following equation:

$$P/P_o = 1 - [1 - \exp(-\Phi/V)] \times V/\Phi \{1 + [(f + g_1)/g_2]^2 \times V/2\Phi\}$$

where f , g_1 and g_2 are rate constants (see Introduction) and $\Phi = (f + g_1)h$, where h is a constant related to the distance over which a cross-bridge may act relative to sarcomere length. Given that Φ approximates to the Hill constant b , the initial value of h was set to the mean value

of b/k_{tr} in pCa 4.5 and then, by trial and error, a value of h obtained (0.02725) such that $(f + g_1)$ most closely matched k_{tr} values at that calcium concentration. Data were fitted to the equation using a non-linear least-squares regression (Solver, Microsoft Excel); the r^2 value for the fit exceeded 0.96 for all data sets.

Data are presented as means \pm s.d. Data were checked for normality using the Shapiro–Wilk test and were normally distributed. Student's paired t tests were used to test for differences between variables at pCa 4.5 compared with pCa 5.6 ($P < 0.05$).

Results

Twenty-five type I fibres were measured at pCa 4.5, and 22 of these were also successfully measured whilst activated at the lower calcium concentration of pCa 5.6. There was a 22% decrease in the isometric force generated in pCa 5.6 solution compared with pCa 4.5 (Fig. 4 and Table 1). Records of shortening under different loads for one fibre at pCa 4.5 and 5.6 are shown in Fig. 3, and it is evident that for a given relative load the velocity of shortening was slower at the lower calcium concentration. Differentiating the length signal showed that the velocity of shortening was constant after 50 ms from the start of the movement in both high- and low-calcium solutions. The full force–velocity relationships of two fibres are shown in Fig. 4, and the contractile properties for all fibres are given in Table 1. In Fig. 4A and C, the decreased isometric force and maximal velocity of unloaded shortening (V_{max}) with

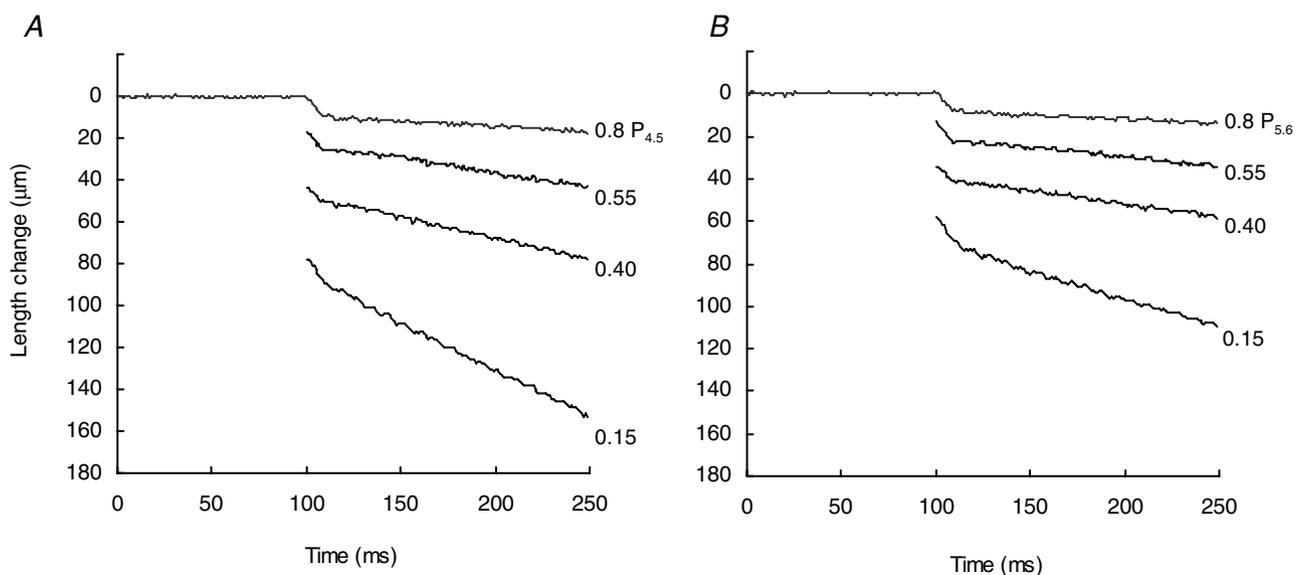


Figure 3. Length traces taken from a series of four isotonic shortening tests at pCa 4.5 (maximal activation; A) and pCa 5.6 (here 89% of P_o at pCa 4.5; B)

The target loads are shown to the right of each trace. The four length traces are superimposed on to each other. Data from the final 100 ms (150–250 ms) of each trace were fitted to a linear regression to provide the slope of the line and thus the velocity of shortening.

pCa		Observed P_o (N cm^{-2})	V_{\max} (FL s^{-1})	a/P_o	k_{tr} (s^{-1})	Peak power (W l^{-1})
4.5	Mean	12.9	0.63	0.113	3.15	4.82
	s.d.	3.2	0.11	0.025	0.40	1.33
5.6	Mean	10.5	0.36	0.165	2.49	2.71
	s.d.	2.9	0.08	0.056	0.35	0.96
Percentage change	Mean*	-22	-44	+47	-23	-46
	s.d.	8	7	31	7	10

Data are for 25 fibres measured at pCa 4.5 and 22 fibres at pCa 5.6. The percentage changes are derived from the 22 fibres tested at both calcium concentrations. Values of V_{\max} and a/P_o were obtained from fitting data to the Hill (1938) equation. * All means different from pCa 4.5, $P < 0.001$.

the lower activating calcium are clearly seen. What is not so evident in Fig. 4A and C is that there was also a change in curvature, but this can be seen when the fitted force–velocity curves are normalized to isometric force and V_{\max} , as in Fig. 4B and D, with the curve determined at the lower calcium concentration being less concave.

On average, maximal power declined from 4.82 ± 1.33 to $2.71 \pm 0.96 \text{ W l}^{-1}$ as a consequence of the decrease in P_o (22%) and V_{\max} by 44%. However, a/P_o , the measure of curvature, increased by 47% (Table 1), which offset some of the changes in power due to the decreases in P_o and V_{\max} . The fraction, M , of P_o or V_{\max} at which maximal

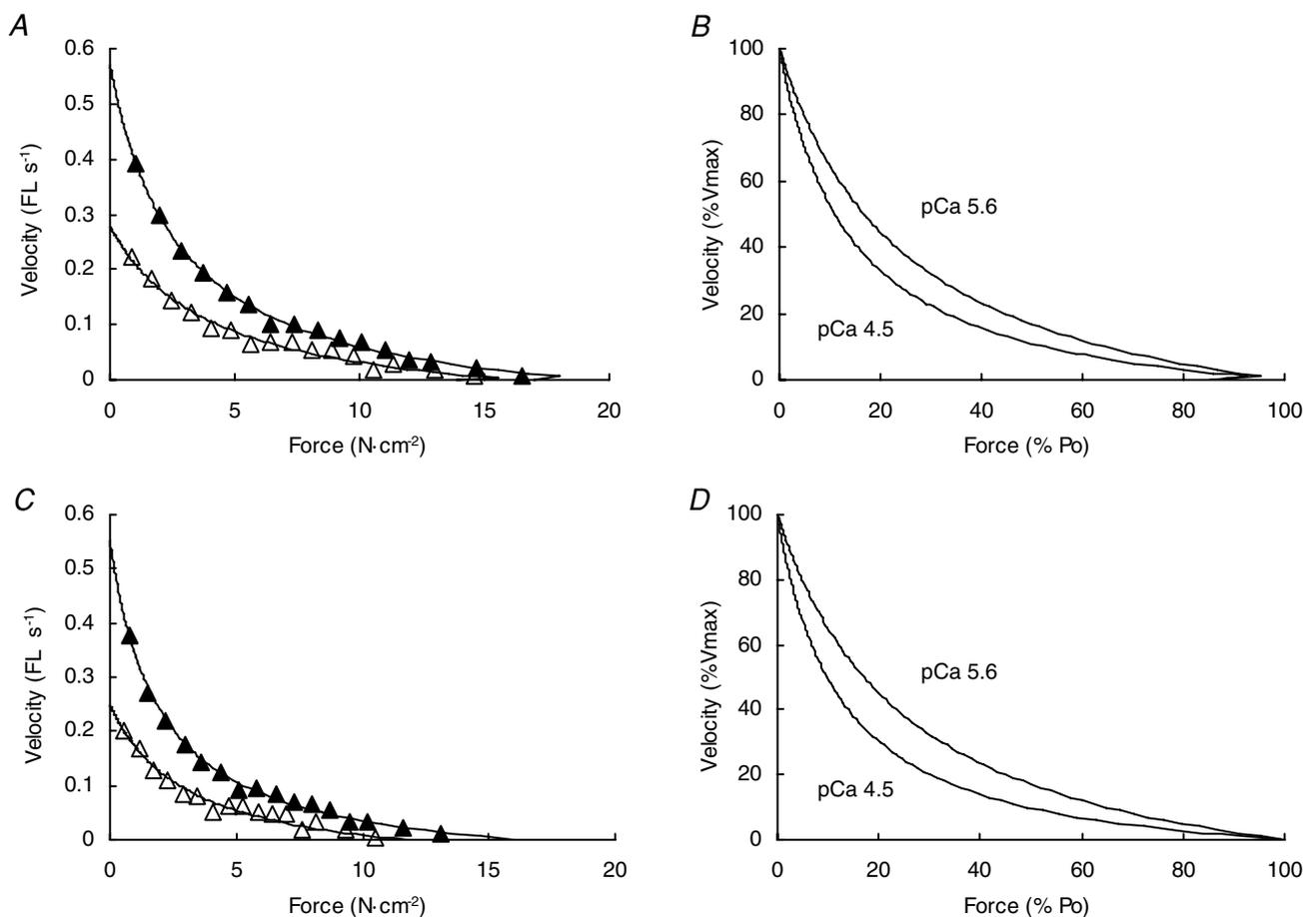


Figure 4 Force–velocity relationships for two fibres measured at pCa 4.5 and 5.6

In A and C, the values measured at pCa 4.5 are shown as filled symbols and those at pCa 5.6 as open symbols. In B and D, the data are normalized to maximal isometric force (P_o) and unloaded shortening velocity (V_{\max}) to illustrate the change in curvature of the relationships.

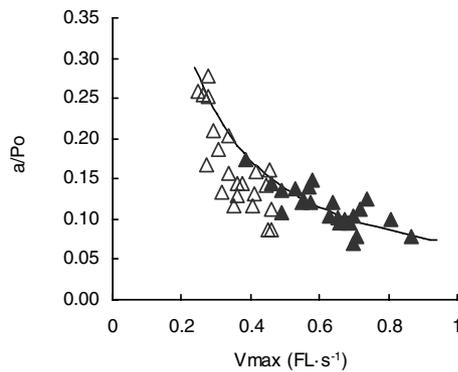


Figure 5. The relationship between a/P_o and V_{\max}
 Filled symbols represent fibres measured at pCa 4.5; open symbols, fibres measured at pCa 5.6. The line shown is $a/P_o = b_{\text{bar}}/V_{\max}$, where b_{bar} is the mean value of the Hill constant b for fibres at pCa 4.5.

power was obtained (Woledge *et al.* 1985) increased from 0.24 at pCa 4.5 to 0.27 at pCa 5.6.

At pCa 4.5 there was a clear tendency for the fastest fibres to have greater curvature of the force–velocity relationship, so there was an inverse relationship between a/P_o and V_{\max} (Fig. 5). When the same fibres were activated at pCa 5.6, they demonstrated lower V_{\max} and higher values of a/P_o , thus appearing to move further along the same relationship as seen between V_{\max} and a/P_o at pCa 4.5.

Figure 6 shows the rate of tension redevelopment following a rapid release and restretch (k_{tr}) for one fibre at pCa 4.5 and pCa 5.6. The k_{tr} declined, on average from just over 3 s^{-1} at pCa 4.5 to approximately 2.5 s^{-1} at pCa 5.6, a decrease of 23% (Table 1).

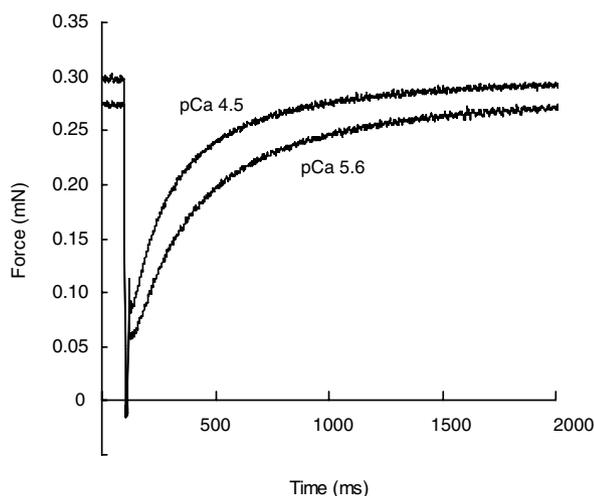


Figure 6. Redevelopment of force following a rapid release and restretch of one fibre activated at pCa 4.5 and pCa 5.6

Discussion

The first objective of the work described here was to quantify the effects of reducing the activating calcium on the force–velocity relationships of skinned type I muscle fibres. A relatively small reduction in activating calcium, from pCa 4.5 to 5.6, was chosen so that reasonable forces were produced to allow the force–velocity relationships to be determined with confidence. The 23% reduction in force and approximate halving of V_{\max} at pCa 5.6 is consistent with other reports for rat slow fibres (McDonald, 2000). Moss (1986) has suggested that there are two phases of shortening, and that the second, slower phase is the more sensitive to the activating calcium concentration. If there were two such shortening phases in the data presented here (Fig. 3) then the values we report will have been for the second phase, since velocity was measured after 50 ms when a steady shortening was established. The decrease in k_{tr} we have observed is also consistent with other observations on mammalian muscle (Brenner, 1988; Metzger & Moss, 1990; Chase *et al.* 1994; de Tombe & Stienen, 2007).

The reduction in peak power as a consequence of reductions in P_o and V_{\max} was offset by the observed decrease in curvature of the force–velocity relationship. The contribution that curvature makes to peak power can be determined from the value of M^2 ; M being the fraction of either P_o or V_{\max} at which maximal power occurs, which is, in turn, derived from a/P_o (Woledge *et al.* 1985). Peak power is given by $M^2 \times P_o \times V_{\max}$. The value of M^2 , calculated from the values of a/P_o in Table 1, increased by 26%, thus the peak power at pCa 5.6 was 26% greater than it might otherwise have been but for the decrease in curvature of the force–velocity relationship.

The second objective was to see how changes in cross-bridge kinetics might account for the changes in curvature. To this end, the force–velocity data were fitted to the two-state cross-bridge model of Huxley (1957). Modelling the data in this way gave values for $(f + g_1)$ and g_2 (Table 2). Plotting the sum of the rate constants $(f + g_1)$ as a function of g_2 (Fig. 7) shows a linear relationship for the fibres measured at pCa 4.5, such that $(f + g_1)$ is lower in the slower fibres with smaller values of g_2 . The relationship has, however, a significant intercept on the ordinate, giving a value of $(f + g_1)$ of about 1 s^{-1} . Consequently, the curvature of the force–velocity relationship, given by dividing $(f + g_1)$ by g_2 , is less for the slower fibres, which is consistent with the observation of higher values of a/P_o for the slower fibres in Fig. 5 and similar observations we have made with human fibres (Gilliver *et al.* 2009). Reducing the calcium concentration to pCa 5.6 decreased both $(f + g_1)$ and g_2 while maintaining a relationship which was similar to that seen at pCa 4.5, again with an intercept giving a value for $(f + g_1)$ of about 0.9 s^{-1} when extrapolated to $g_2 = 0$. The separate relationships between $(f + g_1)$ and

Table 2. Kinetic values for skinned fibres activated with two different calcium concentrations obtained from fitting force–velocity data to the Huxley (1957) model

pCa		V_{\max} (FL s ⁻¹)	$(f + g_1)$ (s ⁻¹)	g_2 (s ⁻¹)	$(f + g_1)/g_2$
4.5	Mean	0.53	3.33	19.41	0.17
	S.D.	0.07	0.45	2.46	0.02
5.6	Mean	0.28	2.41	10.30	0.24
	S.D.	0.04	0.35	1.42	0.03
Percentage change	Mean*	-48	-29	-48	+38
	S.D.	6	8	6	15

Data are for 25 fibres measured at pCa 4.5 and 22 fibres at pCa 5.6. The percentage changes are derived from the 22 fibres tested at both calcium concentrations. * All means different from pCa 4.5, $P < 0.001$.

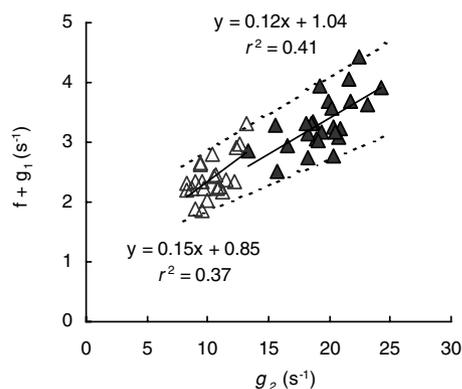
g_2 for fibres activated at pCa 4.5 and 5.6 were statistically indistinguishable from one that combined both sets of data, and it appears, therefore, that reducing the activating calcium simply moves fibre along the relationship that exists between $(f + g_1)$ and g_2 at maximal activation. The intercept on the ordinate is of particular interest, since it is the reason that the curvature of the force–velocity relationship varies both between fibres and with different degrees of activation. Since, in the Huxley formulation, curvature is given by $(f + g_1)/g_2$, it is clear from the data in Fig. 7 that this ratio will tend to a constant value at high values of g_2 whilst for low values of g_2 the ratio rises rapidly. This is consistent with the data shown in Fig. 5.

The kinetic analysis of the force–velocity curves described above suggests that $(f + g_1)$ decreases with a decrease in the activating calcium concentration. This was confirmed by independent measures of the rate constant for the redevelopment of tension following rapid

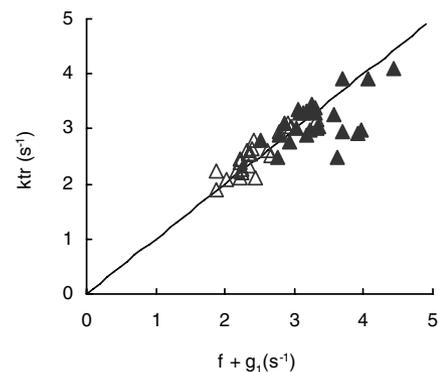
release and restretch (k_{tr}). The absolute values for the rate constants $(f + g_1)$ obtained by fitting the data to the Huxley equation depend on the value of h that is used, and the value of 0.02725 was chosen because it allowed the closest agreement between the values for $(f + g_1)$ and the k_{tr} obtained with maximal activation at pCa 4.5. The same constant was used to fit the data at pCa 5.6 to the Huxley equation. In Fig. 8, the relationship between $(f + g_1)$ and k_{tr} is shown for data obtained at pCa 4.5 and 5.6, and it is evident that both sets of data fall very close to the line of identity. It is not surprising that the data at pCa 4.5 fit the line of identity, since the value of h was specifically chosen to achieve this, but it is reassuring that the same value is applicable to fibres activated at the lower calcium concentration. Consistent with this correlation is the fact that the percentage reduction in $(f + g_1)$ when reducing the calcium (Table 2) was similar to the reduction in k_{tr} (Table 1).

The k_{tr} is considered to be the sum of $(f_{app} + g_{app})$, the rate constants for attachment and detachment in a two-state model of cross-bridge action, and is equivalent to $(f + g_1)$ in the Huxley equation. The decrease in $(f_{app} + g_{app})$ with decreasing activation in skinned fibres has been shown to be due to a reduction in f_{app} while g_{app} remains constant (Brenner, 1988); it seems reasonable to assume, therefore, that the decrease in $(f + g_1)$ that we have observed when decreasing the activating calcium (Figs 7 and 8 and Table 2) is also due to a reduction in f .

The Huxley formulation offers no explanation of why f and g_2 might vary with the activating calcium concentration or, indeed, why the two constants should be related to one another. It has previously been suggested that an accumulation of low-force cross-bridges might lead to a decrease in the apparent rate constant for attachment in fatigued muscle (Jones *et al.* 2006; Jones,

**Figure 7. The relationships between the sum of the rate constants $(f + g_1)$ and g_2**

Fibres were activated with pCa 4.5 (filled symbols) or pCa 5.6 (open symbols). Dotted lines show 95% confidence intervals for continuous line fitted to all data points. Upper bound, $y = 0.124x + 1.612$; and lower bound, $y = 0.086x + 1.001$.

**Figure 8. The relationship between the sum of the rate constants $(f + g_1)$ determined by fitting data for force–velocity curves to the Huxley (1957) model and the rate constant of tension recovery (k_{tr}) following rapid shortening and restretching**

Filled symbols represent fibres measured at pCa 4.5; open symbols, fibres measured at pCa 5.6. The line of identity is shown.

2010), and this might occur with low activating calcium. It is possible that such cross-bridges might also reduce the shortening velocity (Moss, 1986), but the existence of low-force cross-bridges that would provide an effective resistance is doubtful (Linari *et al.* 2004).

In summary, the present study has shown that reducing the activating calcium with type I skinned rat fibres decreases the curvature of the force–velocity relationship in such a way as to reduce the impact of decreases in P_0 and V_{\max} by approximately 25%. A kinetic analysis indicates that the change in curvature is due to linked reductions in the rate constants f and g_2 in the Huxley model. The effects of low calcium on the rate of force development and on the rate of shortening have both been studied before, but generally as separate entities. The present work indicates that the two processes are linked, and this needs to be part of any model of cross-bridge action.

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