Force generation examined by laser temperature-jumps in shortening and lengthening mammalian (rabbit psoas) muscle fibres

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We examined the tension change induced by a rapid temperature jump (T-jump) in shortening and lengthening active muscle fibres. Experiments were done on segments of permeabilized single fibres (length (L_0) ~ 2 mm, sarcomere length $2.5 \mu\text{m}$) from rabbit psoas muscle; [MgATP] was 4.6 mM , pH 7.1 , ionic strength 200 mM and temperature $\sim 9^\circ\text{C}$. A fibre was maximally Ca^{2+} -activated in the isometric state and a $\sim 3^\circ\text{C}$, rapid ($< 0.2 \text{ ms}$), laser T-jump applied when the tension was approximately steady in the isometric state, or during ramp shortening or ramp lengthening at a limited range of velocities (0 – $0.2 L_0 \text{ s}^{-1}$). The tension increased to 2- to $3 \times P_0$ (isometric force) during ramp lengthening at velocities $> 0.05 L_0 \text{ s}^{-1}$, whereas the tension decreased to about $< 0.5 \times P_0$ during shortening at 0.1 – $0.2 L_0 \text{ s}^{-1}$; the unloaded shortening velocity was $\sim 1 L_0 \text{ s}^{-1}$ and the curvature of the force–shortening velocity relation was high (a/P_0 ratio from Hill's equation of ~ 0.05). In isometric state, a T-jump induced a tension rise of 15–20% to a new steady state; by curve fitting, the tension rise could be resolved into a fast (phase 2b, 40 – 50 s^{-1}) and a slow (phase 3, 5 – 10 s^{-1}) exponential component (as previously reported). During steady lengthening, a T-jump induced a small instantaneous drop in tension, followed by recovery, so that the final tension recorded with and without a T-jump was not significantly different; thus, a T-jump did not lead to a net increase of tension. During steady shortening, the T-jump induced a pronounced tension rise and both its amplitude and the rate (from a single exponential fit) increased with shortening velocity; at 0.1 – $0.2 L_0 \text{ s}^{-1}$, the extent of fibre shortening during the T-jump tension rise was estimated to be $\sim 1.2\% L_0$ and it was shorter at lower velocities. At a given shortening velocity and over the temperature range of 8 – 30°C , the rate of T-jump tension rise increased with warming ($Q_{10} \approx 2.7$), similar to phase 2b (endothermic force generation) in isometric muscle. Results are discussed in relation to the previous findings in isometric muscle fibres which showed that a T-jump promotes an early step in the crossbridge–ATPase cycle that generates force. In general, the finding that the T-jump effect on active muscle tension is pronounced during shortening, but is depressed/inhibited during lengthening, is consistent with the expectations from the Fenn effect that energy liberation (and acto-myosin ATPase rate) in muscle are increased during shortening and depressed/inhibited during lengthening.

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The active force in isometric muscle is temperature sensitive (Hadju, 1951) so that, in mammalian muscle, it increases ~ 2 -fold when the temperature is raised from $\sim 10^\circ\text{C}$ to physiological ($> 30^\circ\text{C}$) temperatures (Ranatunga & Wylie, 1983; Ranatunga, 1994). Consequently, a rapid temperature jump (T-jump) induces a rise in force to a level as expected from the steady state experiments, in skinned and intact muscle fibres (Ranatunga, 1996; Coupland & Ranatunga, 2003).

The T-jump force-rise is bi-exponential (labelled phase 2b and phase 3), where the (faster) phase 2b is identified as 'endothermic force generation' in attached cross-bridges (Davis & Harrington, 1987; Goldman *et al.* 1987; Bershitsky & Tsaturyan, 1989, 1992; Ranatunga, 1996). Phase 2b has been compared with a slow component of the (phase 2) quick tension recovery (Huxley & Simmons, 1971) obtained in length-release experiments (Davis & Harrington, 1993; Davis & Rodgers, 1995),

but their exact correspondence, and the structural basis of the T-jump force response, remain unresolved (see Bershtsky & Tsaturyan, 2002; Coupland *et al.* 2005; Ferenczi *et al.* 2005). In isometric muscle fibres, the T-jump force generation has been identified as a molecular step before the release of inorganic phosphate (P_i) by cycling crossbridges, i.e. a transition between two AM.ADP. P_i states (Ranatunga, 1999). Interestingly, other studies on muscle fibres (Fortune *et al.* 1991; Kawai & Halvorson, 1991; Dantzig *et al.* 1992; Ranatunga *et al.* 2002) and on myofibrils (Tesi *et al.* 2000) and also mechano-kinetic modelling (Smith & Sleep, 2004) also have led to a similar conclusion that the force generation occurs prior to P_i release; effects of increased [MgADP] on muscle force could also be accommodated on such a scheme (Coupland *et al.* 2005). Apart from a brief report on shortening muscle fibres (Bershtsky & Tsaturyan, 1990), all the T-jump studies summarized above have been on isometric muscle and there have been no studies in lengthening muscle.

It is well known that the force that a muscle develops varies with the velocity of filament sliding, i.e. during muscle shortening and lengthening. With an increase of shortening velocity, force declines below isometric force (P_0) and reaches zero at the maximum velocity ($V_{\max}$); conversely, an active muscle develops $\sim 2 \times P_0$ as lengthening velocity is increased to $1\text{--}2 L_0$ (muscle fibre length) s^{-1} (Katz, 1939; Lombardi & Piazzesi, 1990). Moreover, the energy production and the acto-myosin ATPase rate in muscle are increased with shortening and decreased with lengthening, a cardinal principle of muscle contraction that is commonly referred to as the Fenn effect (Fenn, 1924; Curtin & Davies, 1973; He *et al.* 1999; Linari *et al.* 2003b).

On the basis of the above, it would be of interest to examine the T-jump-induced force generation in shortening and lengthening muscle fibres; this was the aim of our study. Thus, we have examined in maximally activated single psoas fibres the force responses induced by a small, rapid ($\sim 3^\circ\text{C}$ in < 0.2 ms) T-jump at $8\text{--}9^\circ\text{C}$ when the fibre is shortening or lengthening at different velocities. Our results show that, compared with that in isometric muscle, the T-jump force rise is enhanced during shortening and depressed during lengthening, basically demonstrating the Fenn effect. Preliminary data from this study have been reported to the European Muscle Conference and published in abstract form (Ranatunga *et al.* 2006).

Methods

Fibre preparation and buffer solutions

Adult male rabbits were killed by an intravenous injection of an overdose of sodium pentobarbitone and other tissues were harvested for different experiments by other

researchers. Fibre bundles from the psoas muscle were prepared and chemically skinned using 0.5% Brij 58, as previously described (Fortune *et al.* 1989). The buffer solutions contained 10 mM glycerol-2-phosphate (a temperature-insensitive pH buffer; $\text{pH} = 7.1$), 4.6 mM MgATP, 12 mM creatine phosphate, 15 mM EGTA (relaxing solution), CaEGTA (activating solution) or HDTA (pre-activating solution), 9 mM glutathione and ~ 50 mM potassium acetate. The solution compositions were calculated using a computer program (provided for us by Professor Mike Ferenczi, Imperial College, London) for solving multi-equilibria, maintaining ionic strength (at 200 mM) and a free $[\text{Ca}^{2+}]$ in the activating solutions of ~ 0.032 mM. Creatine kinase ($1\text{--}2$ mg ml^{-1} ; ~ 300 units mg^{-1} , Sigma) was added to buffer solutions before use to decrease [MgADP]. All solutions also contained 4% Dextran (mol. mass ~ 500 kDa) in order to compress the filament lattice spacing to normal dimensions (Maughan & Godt, 1979).

Apparatus and laser T-jump technique

Details of the trough assembly, design of the force transducer (natural resonant frequency, 14 kHz) and other aspects of the experimental apparatus have been described in detail previously (Ranatunga, 1996, 1999; Coupland *et al.* 2001). Briefly, the trough assembly was mounted on the stage of an optical microscope and the fibre could be moved and immersed in the different trough solutions (relaxing, pre-activating and activating solutions), by means of a lever mechanism. Using thermo-electric modules (Peltier units) fixed on the back wall, and with feedback from a small thermistor, the solution temperature in the front experimental trough could be changed and/or clamped at a desired pre-T-jump temperature and was monitored with a thermocouple. In order to maintain fibre stability, the temperature in the other troughs containing relaxing and pre-activating solutions was kept below 5°C by cooling fluid circulating through the trough assembly.

The details of the technique of laser pulse-induced T-jumps in muscle fibres have been previously described (Ranatunga, 1996, 1999). In most of the present experiments, the temperature of the front experimental trough was clamped at $8\text{--}9^\circ\text{C}$ and a T-jump was induced by a 0.2 ms laser pulse radiation ($\lambda = 1.32 \mu\text{m}$) from a Nd-YAG laser (Schwartz Electro-Optics) that entered the experimental trough through the front glass window. A laser pulse heated the buffer solution in the trough and the muscle fibre bathed in it; the heated fibre/heated fluid volume ratio was > 2500 . A fine thermocouple placed near the fibre showed that, after a laser pulse, the elevated solution temperature remained constant for at least ~ 500 ms and decreased only slowly (half-time longer than 5 s) afterwards (see Fig. 1 of Ranatunga, 1996).

Experimental protocols and data analysis

Using dark-field illumination on the stage of a binocular microscope, single fibres were dissected under paraffin oil. A short fibre segment was attached using nitrocellulose glue between two metal hooks, one connected to the force transducer and the other to the motor. The sarcomere length was set to $2.5\ \mu\text{m}$ using He–Ne laser diffraction and the fibre width and length were measured under light microscopy; fibre length (L_0) was $\sim 2\ \text{mm}$ in most experiments. The force responses to a standard T-jump of 3°C were examined after a fibre was activated to steady isometric state and then exposed to steady shortening

and lengthening at different velocities. A T-jump was applied 200–500 ms after the beginning of a ramp shortening/lengthening when the force during filament sliding reached a steady level; this was approximately so for most shortening velocities, so that the pre-T-jump tension represented the steady shortening tension without a T-jump. With lengthening, however, there was a continued slow tension rise and the tension level without a T-jump was estimated by a curve (exponential) fitted to the late pre-T-jump tension trace. In some experiments, tension records were made with and without T-jumps at the same velocities (see Figs 1 and 2). Fibres were regularly examined

Figure 1. Tension responses to lengthening, shortening and T-jump from one fibre

The fibre held isometric was maximally Ca^{2+} activated at $\sim 9^\circ\text{C}$ and, during the steady isometric tension plateau (P_0), a ramp lengthening (A, $\sim 0.06 L_0 \text{ s}^{-1}$; B, $\sim 0.1 L_0 \text{ s}^{-1}$) or a ramp shortening (C, $\sim 0.05 L_0 \text{ s}^{-1}$; D, $\sim 0.065 L_0 \text{ s}^{-1}$) of $\sim 5\% L_0$ was applied; dotted lines indicate the isometric base levels. The tension rises above P_0 during lengthening and falls below P_0 during shortening to approximately steady levels. The bottom trace in each frame is the position of the first order diffraction (sarcomere length, SL (μm)) from a He–Ne laser beam that passed through a $\sim 0.5\ \text{mm}$ length of the fibre; the signal does not indicate marked changes due to a T-jump. As shown by the thermocouple record (the second trace from the top in each frame) in B, C, D and E, a T-jump of $\sim 3^\circ\text{C}$ was induced by a $< 0.2\ \text{ms}$, near-infrared, laser pulse applied to the fibre and the solution bathing it; the initial peak and slow decay are due to direct heat absorption by the thermocouple (Goldman *et al.* 1987). E shows the tension response to an identical T-jump when the fibre was held isometric. Note that when the fibre is isometric (E) or shortening (C and D), a T-jump induced a tension rise to a new steady level; on the other hand, during ramp lengthening when the tension rises to a new level, a T-jump produces little tension rise.

under the microscope and an experiment was terminated when evidence of fibre damage was observed.

The outputs from the force transducer, the motor (fibre length), the thermocouple and, in some experiments, the diffractometer (sarcomere length change) were examined on two digital cathode ray oscilloscopes and digital voltmeters and, using a CED micro-1401 (Mk II) laboratory interface and Signal 2/3 software (Cambridge Electronic Design Ltd, Cambridge, UK), stored in a PC based computer. The sampling rate was 15 kHz and the duration of a sweep examined was 1 s. In some experiments, the position of the first order He–Ne laser diffraction was also monitored by means of a position detector and the output of this diffractometer was used to monitor sarcomere length change during imposed fibre length changes; the analyses will be made with regard to fibre length (L_0) and the corresponding sarcomere length will be briefly mentioned. A continuous record of tension

and temperature was also made on a chart recorder. The force measurements and curve fitting to tension records were made using Signal software and further analyses were made using Fig.P software (Biosoft, Great Shelford, Cambridgeshire, UK).

Simulating T-jump transients

To qualitatively model the changes in T-jump-induced force responses in shortening fibres, we used the same 5-step scheme for the crossbridge/AM–ATPase cycle as used previously for isometric muscle fibres (Coupland *et al.* 2005); the aim was to see whether the basic trends observed in experiments can be seen in such simulations. The scheme is given below (Scheme 1) and shortening at a certain velocity was simulated by increasing k_{+4} (i.e. the ADP release step – see He *et al.* 2000).

Figure 2. Analyses of the T-jump tension responses

A, five superimposed tension records (middle panel) and corresponding length records (bottom panel) from an experiment. The fibre was held isometric and maximally Ca^{2+} activated at $\sim 9^\circ\text{C}$ and, when the active tension had reached a plateau, a T-jump of $\sim 3^\circ\text{C}$ (schematically shown in the top panel) was applied. The tension trace marked 'isometric' in middle panel was so obtained; the tension rises to a new steady level following the T-jump and a double exponential curve is fitted (dotted line on the right-hand of the trace) to the post-T-jump tension trace. Once the temperature was clamped again at $8\text{--}9^\circ\text{C}$, it was stretched at a constant velocity of $\sim 50\text{ nm hs}^{-1}$ (hs, half-sarcomere) (top length record in the bottom panel) to obtain the two tension traces marked 'lengthening' (one without and the other with a standard T-jump). Note that, during lengthening, the tension rose towards a higher steady level ($\sim 2.2 P_0$) and that the T-jump does not lead to a net increase of tension; the instantaneous drop in tension due to T-jump is more obvious in lengthening fibre and this may indicate expansion in non-crossbridge elements (Ranatunga, 1996). The procedure was then repeated when the fibre was shortening at a similar velocity (lower length record in the bottom panel) to obtain bottom two tension traces marked 'shortening'. The tension decreased towards a steady level below the isometric tension ($\sim 0.5 P_0$) during steady shortening, but the standard T-jump induced a pronounced tension rise; the 300 ms duration of the post T-jump tension trace is fitted with a single exponential function. **B**, traces from another fibre where the presentation is similar to **A**, but separate tension records without T-jump were not made. During shortening, the tension immediately before T-jump represents steady tension level at the pre-T-jump temperature. For lengthening, single exponential curve was fitted to the pre-T-jump tension trace and to the post-T-jump trace (dotted lines through the trace) and, by extrapolation to the ramp end, the tension change induced by a T-jump was determined.

The other features were as described before; briefly, the forward rate constant of Step 1 (k_{+1}) is temperature-sensitive (endothermic) force generation. Step 2 is rapid P_i release and steps 3 and 4 represent the two-step ADP release (slow in isometric muscle). Step 5 includes all the steps after ADP release that are necessary to re-prime a crossbridge for the next cycle including the $M.ADP.P_i \leftrightarrow M.ATP$ cleavage step after detachment, as indicated, but not separately identified in the model; the overall rate through this route is low (k_{+5} , $\sim 10 \text{ s}^{-1}$; see He *et al.* 2000) and was forward biased. The states $AM^*.ADP.P_i$, $AM^*.ADP$ and $AM^*.ADP$ were taken to be equal-force bearing states (F) and the sum of their fractional occupancy is taken as force (see Coupland *et al.* 2005 for other details). The linear kinetic Scheme 1 above was solved by the matrix method using Mathcad 2000 Professional software (Mathsoft) as previously described (Gutfreund & Ranatunga, 1999). After a steady state in the occupancy is reached, the method was used to simulate a T-jump relaxation by increasing k_{+1} with a Q_{10} of 4 as previously described (see Ranatunga, 1999; Coupland *et al.* 2005) and the approach to the new steady state obtained.

Scheme 1

The data presented here are from experiments on 16 single muscle fibres; their mean ($\pm$ s.e.m.) isometric tension (P_0) at $\sim 9^\circ\text{C}$ was $192 (\pm 16) \text{ kN m}^{-2}$, the fibre length (L_0) was $1.33 (\pm 0.08) \text{ mm}$; in data presentation, the velocity and length changes will be given in fibre length (L_0) and their relation to half-sarcomere length will be considered later.

Results

Tension responses to T-jumps during ramp shortening and lengthening

The tension response induced by a standard laser T-jump of $\sim 3^\circ\text{C}$ was examined in maximally Ca^{2+} -activated muscle fibres, when they were held isometric and when they were steadily shortening or lengthening at different constant velocities; Fig. 1 shows tension responses from one experiment. Figure 1A and B show that during ramp lengthening, the tension rises rapidly towards a level of about twice the isometric tension (P_0) and a T-jump produces no further tension rise; a small instantaneous drop in tension was sometimes seen (as in Fig. 1B), indicative of thermal expansion in the fibre (see Ranatunga, 1996). Figure 1C and D show that, during

steady shortening, the tension decreases to a level lower than P_0 , the tension level reached being lower at the higher velocity (D); in either case, a T-jump produces a marked tension rise with a characteristic time course. As reported in previous studies, a T-jump induces a rise in tension to a new steady level when the fibre is held in isometric (Fig. 1E).

The superimposed tension traces shown in Fig. 2A and B illustrate the type of analyses carried out to characterize the T-jump tension responses. In isometric state, the post-T-jump tension trace could be fitted with a double exponential curve, as reported in previous studies (see Ranatunga, 1999), yielding the time constant and the amplitude of two components (referred to as fast, phase 2b and slow, phase 3; see Ranatunga *et al.* 2002); at the post-T-jump temperature of $11\text{--}12^\circ\text{C}$, their reciprocal time constants were $\sim 50 \text{ s}^{-1}$ (phase 2b) and $5\text{--}10 \text{ s}^{-1}$ (phase 3). Since, the pre-T-jump tension had reached an approximate steady level during ramp shortening, the post-T-jump tension rise could be characterized, as in the isometric case, by curve fitting; however, the T-jump tension rise during shortening required only a single exponential, with a reciprocal time constant of $20\text{--}30 \text{ s}^{-1}$ at the velocities shown in Fig. 2. During ramp lengthening, the tension rose rapidly initially and continued to rise slowly afterwards, as has been observed previously (see Pinniger *et al.* 2006). The fact that a T-jump does not lead to a net tension rise was, nevertheless, clear when tension records at the same velocity, with and without a T-jump, were examined (Fig. 2A); this was not experimentally profitable, however, when the aim was to collect data from a fibre at a range of different velocities. Therefore, whether a T-jump induced a net tension change was determined by fitting a single exponential curve each to the pre-T-jump tension record and to the post-T-jump tension record (see Fig. 2B) and determining the difference in the extrapolated tensions at the ramp-end.

The velocity dependence of the T-jump tension response

The amplitude of tension change and the rate of the exponential components, derived by curve fitting to the T-jump-induced tension responses, were obtained at different velocities in six fibres and the data are shown in Fig. 3. Figure 3A shows that, plotted as a ratio of the post-T-jump tension, the amplitude of the T-jump-induced tension rise in shortening fibres is correlated with velocity (filled circles, $P < 0.05$); the amplitude of tension change during lengthening is not significantly different from zero and is not correlated with velocity ($P > 0.05$). Figure 3B (filled circles) shows that the rate of T-jump-induced tension rise during steady shortening increases with velocity ($P < 0.001$). For completeness, the data for the post-T-jump tension rise

during steady lengthening are also shown in Fig. 3B (open circles). Since the amplitude is negligible (Fig. 3A), this is only an apparent rate and it increases slightly with velocity; the rate of tension rise determined by curve fitting to the late part of the pre-T-jump tension trace (crosses) was not significantly different from the post-T-jump rates (Student's *t* test, $P > 0.05$). As found previously, the T-jump-induced tension rise in the isometric case contained two components; their mean ($\pm$ s.e.m.) amplitude and rates ($40\text{--}50\text{ s}^{-1}$ and $5\text{--}10\text{ s}^{-1}$ in the present experiments) are shown by open squares on the ordinates. Thus, the data in Fig. 3 show that the tension during steady lengthening is not changed by a T-jump, whereas the tension in shortening is enhanced by a T-jump – as found in previous studies on isometric fibres.

The force–velocity curve

Determination of the steady-state force–velocity curve was not a main concern in this study; nevertheless, it would be useful to survey the force–velocity range

covered in the above experiments, particularly in relation to the maximum or unloaded shortening velocity. In experiments on six fibres, we determined the unloaded shortening velocity (V_0) at $8\text{--}9^\circ\text{C}$, by the slack test method (Edman, 1979). Figure 4A shows tension responses to different amplitude length steps; the time to tension redevelopment at five different length step amplitudes was measured and V_0 was determined from the slope of a linear regression fitted to such data (Fig. 4B). The mean ($\pm$ s.e.m.) V_0 was $1.17 (\pm 0.2) L_0\text{ s}^{-1}$. Our previous studies (Ranatunga, 1984; Coupland *et al.* 2005) showed that $V_{\max}$ of mammalian, intact and skinned, fast fibres at 10°C would be $1\text{--}2 L_0\text{ s}^{-1}$ and several other studies on fast (type 2) skinned fibres at 12°C (Bottinelli *et al.* 1996; He *et al.* 1999, 2000; Linari *et al.* 2003a) also report $V_{\max}$ and V_0 values ranging from ~ 1 to $2.5 L_0\text{ s}^{-1}$. Given the variability seen, a value of $\sim 1 L_0\text{ s}^{-1}$ for V_0 for fast fibres at $8\text{--}9^\circ\text{C}$ is consistent with those findings. In plots such as Fig. 4B, the intercept value on the ordinate was $0.025 \pm 0.0025 L_0$ (mean $\pm$ s.e.m., $n = 6$); this is slightly higher than $0.021 L_0$ obtained in similar experiments on intact frog fibre (Edman, 1979).

Figure 3. The velocity dependence of the T-jump tension response

Data from 6 fibres in each of which tension responses to a standard T-jump (as in Figs 1 and 2) were collected at different velocities; velocity is plotted as $L_0\text{ s}^{-1}$ on the abscissa, with negative for shortening and positive for lengthening. *A*, amplitude of tension change: the net tension change after a T-jump (post-T-jump minus pre-T-jump tension) is plotted as a ratio of the post-T-jump tension. The T-jump-induced tension rise during shortening (filled circles) is correlated with velocity ($P < 0.05$); the mean ($\pm$ s.e.m., $n = 7$ or 8 per point, total = 57) are plotted and the curve is fitted by eye. During lengthening, the amplitude of tension change by a T-jump (open symbols) is not correlated with velocity ($P > 0.05$) and is not significantly different from zero; individual data ($n = 38$) are plotted. Mean (s.e.m., $n = 30$) for isometric state is plotted at zero velocity (on the ordinate). *B*, rate of tension rise: a single exponential function was fitted to define the post-T-jump tension trace in shortening and the reciprocal of the time constant (τ) is plotted as a rate of tension rise on the ordinate; the rate (filled circles) is correlated with shortening velocity; mean ($\pm$ s.e.m.) are shown where the line is the calculated linear regression for individual data ($P < 0.001$). A similar curve-fitting procedure was also done on tension traces during lengthening, although the T-jump-induced tension amplitude was insignificant (Fig. 3A); the apparent rate of post-T-jump tension rise is very low (mean $\sim 5\text{ s}^{-1}$, open circles, individual data); the crosses show the rate of tension rise determined by curve fit to the late part of pre-T-jump tension trace during lengthening, and they are not significantly different from the post-T-jump rates (Students *t* test, $P > 0.1$). As found previously (Ranatunga, 1999), the T-jump-induced tension response in isometric case contained two components, with rates (reciprocal time constants from bi-exponential curve fit) of $40\text{--}50\text{ s}^{-1}$ and $5\text{--}10\text{ s}^{-1}$ in the present experiments; the mean (s.e.m., $n = 30$) values are shown as squares at zero velocity.

Figure 4C shows, from the T-jump experiments, the pooled 'steady' tension data for different velocities of shortening (filled symbols) and of lengthening (open symbols), at 8–9°C (pre-T-jump temperature), where the curves were fitted by eye. The velocity range is zero (isometric) to $\sim 0.17 L_0 s^{-1}$, or $\sim 0.15 V_0$, for shortening and lengthening. The data show that the tension increases rapidly with an increase of lengthening velocity, whereas the tension decreases more slowly with an increase of shortening velocity. The data examination also showed that the shortening velocity range nearly covers the increase with velocity (the ascending limb) of the bell-shaped, mechanical power (force $\times$ velocity) *versus* shortening velocity curve. The tension during lengthening was estimated here as the tension at the ramp-end (ramp

amplitude was $\sim 5\% L_0$; Fig. 2). However, after an initial rapid rise, the tension continued to rise slowly (at $\sim 1 P_0 s^{-1}$ at $0.1 L_0 s^{-1}$) during a ramp stretch; hence, to be consistent with previous studies (see Getz *et al.* 1998; Pinniger *et al.* 2006), the tension at the transition from the initial (fast) phase to the late (slow) phase of tension rise was also estimated, thus removing the slow tension rise contribution; with that analysis, the force level reached, at the transition and with an increase of lengthening velocity, was $\sim 2 \times P_0$, less than in Fig. 4C. A slow continued tension change (decline) was seen during ramp shortening (see Roots *et al.* 2007) but its amplitude, in the present experiments, remained small ($< 0.2 P_0 s^{-1}$); nevertheless, this uncertainty may have contributed to some of the scatter in the amplitude data analysis in Fig. 3A.

Figure 4. The force–velocity relation at low temperatures (8–9°C)

A, tension responses (upper) to rapid (~ 1 ms) length steps of different amplitudes (lower) to induce fibre slack (horizontal dotted line); the duration of slack time (time delay to tension rise) was measured. B, a plot of the length step amplitude *versus* time delay; the slope of the fitted linear regression gives an unloaded velocity (V_0) of $\sim 1.0 L_0 s^{-1}$. C, from the T-jump experiments, the velocity dependence of steady force at 8–9°C (the pre-T-jump temperature). The data are from 6 fibres, as in Fig. 3 – but include measurements without T-jumps, in each of which data were obtained for both shortening (filled) and lengthening (open) limbs; a symbol represents the mean ($\pm$ S.E.M.) of 5–8 measurements ($n = 68$ for shortening and $n = 46$ for lengthening) and the line through the data was fitted by eye. Note that the force during lengthening is estimated as tension at the ramp-end (ramp amplitude was $\sim 5\% L_0$) – see Fig. 2 legend. D, the mean V_0 ($\pm$ S.E.M., diamond) from 6 fibres and the force shortening velocity data from two of them (circles and squares). An approximate Hill curve is fitted with an a/P_0 ratio of 0.05 (see text).

The fact that, in the shortening limb of the F – V relation (F – V s relation) in Fig. 4C, the force decreases to $< 0.5 P_0$ as the shortening velocity is increased to $\sim 0.1 L_0 s^{-1}$ suggests a high curvature for the F – V s relation, since V_0 is around $1 L_0 s^{-1}$. The temperature-dependence studies on intact mammalian fast and slow muscles, indeed, showed that the curvature of the F – V s relation would become higher (a/P_0 ratio lower) as the temperature is reduced from 30–35 to 10°C (Ranatunga, 1984). Therefore, in experiments on two fibres at 8–9°C, we obtained data for a fuller F – V s relation and they are shown in Fig. 4D; the curve drawn is a fit to a Hill (1938) hyperbolic equation ($P/P_0 = a(V_{\max} - V)/(V + b)$, where P is force, V is velocity and a and b are constants) with an a/P_0 ratio of 0.05; the low ratio indicates a high curvature. Such a high curvature, as obtained here, is also evident in the data reported for skinned fast (type 2) fibres at 12°C, in other previous studies (a/P_0 ratios of 0.05–0.07, Bottinelli *et al.* 1996; Linari *et al.* 2003a). The data in Fig. 4D confirm that, at 8–9°C, the fibre force declines to $< 0.5 P_0$ at shortening velocities of around $0.1 L_0 s^{-1}$ and the curvature of the F – V s relation is high; the high curvature would also account for the variability in the estimates for $V_{\max}$ obtained in studies on mammalian muscle fibres at the low temperatures.

T-jump tension response during ramp shortening

(i) The amplitude of tension rise. A constancy of the amplitude of the tension response to a standard T-jump

Figure 5. Characteristics of the T-jump tension response during shortening

Data during shortening in Fig. 3A are re-analysed; shortening velocity is plotted as $L_0 s^{-1}$ on the abscissa. Open symbols represent individual data ($n = 57$) and the filled symbols, the mean ($\pm$ S.E.M., $n = 7$ or 8 per symbol). The amplitude of tension rise after a T-jump, when normalized to the pre-T-jump isometric tetanic tension, is not significantly correlated with velocity ($P > 0.05$); the mean is shown by the dashed line ($0.24 P_0$). The mean (S.E.M., $n = 30$) amplitude for isometric state, represented in the same manner, is plotted at zero velocity ($0.17 P_0$).

was observed when isometric force was changed by other means in previous experiments (see Discussion). Thus, in Fig. 5, the amplitude of T-jump tension rise is plotted as a ratio of the pre-shortening isometric tension of each fibre; the data show some scatter and they are not correlated with shortening velocity, with a mean of $\sim 25\%$ of isometric force. Thus, the increase in the amplitude obtained in Fig. 3A is due to the shortening velocity-dependent change (decrease) of the post-T-jump force and, in a given fibre, a standard T-jump induces the same, 'absolute', increment of force at different shortening velocities.

(ii) At different times, during approach to the steady state. The above results show that, at steady state, the tension during a ramp shortening is particularly sensitive to a T-jump (Fig. 3A). Figure 6A shows superimposed tension records from an experiment in which a standard T-jump was applied at different times after the onset of the same ramp shortening; a tension record to the same T-jump without shortening (isometric) is also shown. It seems that a T-jump does not induce a marked tension rise, when applied at the beginning of the ramp shortening and that the effect of a T-jump becomes prominent when it is delayed and as the pre-T-jump tension reaches a new steady level during shortening. Figure 6B shows a simple analysis made from two such experiments where the top panel shows the decrease of (pre-T-jump) tension as the ramp shortening progresses with time; the tension approaches a steady level at ~ 500 ms after the beginning of the ramp. The lower panel shows that the post-T-jump (peak) tension increment is small at the beginning of a ramp but increases to a steady maximum of 20–25% when the T-jump is delayed to 500 ms. Given that T-jump produces a clear tension increment in isometric state, the above data indicate the interactive effect of the tension changes during T-jump and ramp shortening. Thus, as shown in Fig. 6C, when the tension change due to shortening is subtracted, the difference tension traces suggest that a T-jump induces a similar tension rise at different times during shortening; to what extent this behaviour is dominated by series compliance remains uncertain.

(iii) At different temperatures. In the above experiments, the T-jump tension responses during shortening were examined at around 10°C. In mammalian muscle, the isometric tension at this temperature is $\sim 50\%$ of that at the body temperatures ($> 30^\circ\text{C}$) and the relative effect of a standard T-jump on isometric tension decreases as the temperature is raised (Ranatunga, 1996; Coupland & Ranatunga, 2003); moreover, the F – V s relation is temperature sensitive so that $V_{\max}$ increases with a Q_{10} of > 2 and the curvature decreases (Ranatunga, 1984) in warming. Therefore, in four experiments, we examined the

T-jump tension responses during shortening at a range of temperatures.

Figure 7A shows tension responses recorded from a fibre at four different temperatures; the fibre was first activated at 7–8°C, and the temperature raised (and clamped) by the Peltier, thermo-electric module system in the trough. At each temperature, it was shortened at the same ramp velocity and a T-jump of 3°C applied when tension during shortening was nearly steady. It is seen that the tension before the ramp (isometric) as well as the tension during shortening, before and after the T-jump, rise with increase of temperature. Figure 7B shows the pooled data (mean $\pm$ S.E.M.) for isometric tension (filled symbols) and for steady-shortening tension (open symbols), plotted against the reciprocal absolute temperature; the temperature dependence of isometric

tension is essentially similar to previous findings, and the data also show that the tension during shortening, at about the same velocity, shows a similar temperature dependence.

Figure 8 shows the pooled data for the rate (upper panel, open symbols) and the amplitude (lower panel) of the tension rise induced by a T-jump, during shortening (velocity range 0.06–0.09 L_0 s⁻¹ – in different experiments), at different initial temperatures; the abscissa is the post T-jump temperature, plotted as reciprocal absolute temperature and the rate is plotted on a logarithmic ordinate. In warming from 10 to 30°C, the rate increases with a Q_{10} of 2.64 and the relative amplitude decreases sharply. The isometric fibre data (filled circles), which are essentially similar to the more detailed data in previous studies (Ranatunga, 1996; Coupland *et al.* 2005),

Figure 6. Tension response to a T-jump at different times during a ramp shortening

A, 8 superimposed tension responses (upper panel) each with a standard T-jump of 3°C applied at a certain time interval; the fibre was activated to steady state at 9°C, shortened at 0.06 L_0 s⁻¹ (lower panel). Note that the uppermost T-jump tension response (ninth) was isometric and the pre-T-jump isometric P_0 level is indicated by the horizontal dashed line. B, tension data from two experiments. Upper panel shows the pre-T-jump tension (as a percentage of P_0) plotted against time (dashed vertical line denotes the beginning of ramp shortening); tension decreases to a steady level at ~500 ms. Lower panel shows the post-T-jump peak tension increase above the pre-T-jump level: the triangle shows the mean ($\pm$ S.E.M., $n = 7$) T-jump-induced isometric tension increment. Note that the apparent T-jump-induced net increment of tension is small at short intervals but increases to a steady level at 500 ms (curves through the data points are fitted by eye). C, the top trace is the isometric T-jump tension response, the middle trace is the ramp-shortening tension record without a T-jump; the lower traces are difference tension traces, obtained by subtracting the middle shortening tension record from each of seven shortening-tension records with a T-jump, applied (as in A) at various times during the ramp shortening. Note that a T-jump induces approximately the same tension rise; the one at the beginning of the ramp, however, seems more similar to the isometric (bi-component).

show that the rate of the faster (phase 2b) exponential component also has a high Q_{10} (~ 3) (see figure legend for details). The temperature dependence of tension during shortening would be influenced by a number of factors; the decrease in the relative amplitude with warming (Fig. 8, lower panel), in particular, is as expected from Fig. 3A. For instance, the relative velocity (V/V_0 , at each temperature) is $0.05\text{--}0.08 V_0$ at $\sim 10^\circ\text{C}$, whereas due to an increase of $V_{\max}$ on warming (Ranatunga, 1984), it would only be $\sim 0.02\text{--}0.04 V_0$ at 20°C ; from Fig. 3A, the relative tension increment by T-jump would hence be lower.

Discussion

The present study extends our previous findings, on the effects of temperature and T-jumps on isometric muscle fibres (Ranatunga, 1996), to shortening and lengthening muscle fibres. We examine below whether our findings provide some insight into the underlying processes that contribute to the generation and maintenance of muscle force under different conditions.

Endothermic force generation in muscle

As summarized in the introduction, previous studies on isometric muscle fibres have shown that, at $\sim 10^\circ\text{C}$,

a T-jump of $3\text{--}5^\circ\text{C}$ induces a tension rise to a new steady level (see Coupland *et al.* 2005); the tension rise consisted of two exponential components, phase 2b (fast) and phase 3 (slow), where phase 2b was thought to represent endothermic force generation in attached crossbridges; phase 2b rate increased markedly with warming (Q_{10} of 3; Ranatunga, 1996) whereas phase 3 rate was much less temperature sensitive. The endothermic force generation represents an early step (before the release of inorganic phosphate) in a Lymn & Taylor (1971) type of scheme for the acto-myosin ATPase pathway in muscle (see Ranatunga, 1999; Coupland *et al.* 2001). The occurrence of a single endothermic force generation step in the crossbridge cycle could basically account for the increase of steady isometric muscle force with warming, the sigmoidal relation between isometric force and temperature, and the effect of inorganic phosphate and of Mg-ADP on isometric force at different temperatures (see references in Coupland *et al.* 2005). Unlike in isometric muscle, the T-jump-induced tension transient in steadily shortening muscle consists of only one exponential component; its relative amplitude is more pronounced and the rate is intermediate between phase 2b and phase 3 rates of isometric muscle (Fig. 3). In the isometric case, the slow, phase 3, component is P_i insensitive and is probably due to a slow, later step in the cycle. During shortening, the

Figure 7. T-jumps during steady shortening at different temperatures

A, superimposed tension (upper panel) and length (lower panel) records from an experiment in which the response to a 3°C T-jump (upward arrow), during same ramp shortening (velocity $\sim 0.06 V_0$), was recorded at a number of different temperatures; the post-T-jump temperature is given to the right of each trace. The isometric tension (i.e. tension before the downward arrow) and the tension during steady shortening are higher and the T-jump-induced tension rise, while shortening, becomes faster at the higher temperatures. B, the temperature dependence of the isometric tension (filled symbols) and of the tension during steady shortening (open symbols). Mean ($\pm$ S.E.M.) data are from 4 fibres in each of which tensions are plotted as a percentage of its isometric tension at the low temperature ($7\text{--}9^\circ\text{C}$); the abscissa is reciprocal absolute temperature ($10^3 \times 1/T$) and is also labelled in $^\circ\text{C}$. The total number of measurements was 42 for isometric ($n = 2\text{--}5$ per data point) and 72 for shortening ($n = 8$ per data point). The shortening (velocity range $0.05\text{--}0.08 V_0$ in different experiments) decreased the tension during steady shortening to $\sim 50\% P_0$ at $\sim 9^\circ\text{C}$. Note that in warming over this temperature range, the isometric tension increases ~ 2 -fold (as shown in previous studies, see references in Coupland *et al.* 2005) and the tension during steady shortening, within this velocity range, also shows a similar increase with warming.

last step (detachment) occurs faster than in isometric – hence, a decreased occupancy of a later stage may lead to a simpler (single exponential) T-jump response. Increase of the rate with shortening velocity is expected from a faster cycle leading to an increase of pre-stroke crossbridges. The difference between $40\text{--}50\text{ s}^{-1}$ in isometric phase 2b and $\sim 20\text{--}30\text{ s}^{-1}$ in shortening is not large (~ 2 -fold), but accounting exactly for such a difference is difficult; moreover, results in Fig. 3B suggest that, at faster shortening velocities, the rate of T-jump force rise may be even higher than in isometric, but this needs to be confirmed experimentally. The slightly higher phase 2b rate in the isometric case is probably due to the slow flow through the cycle, so that the reverse steps in the cycle would contribute more to a relaxation resulting from a rapid perturbation (e.g. T-jump). Another possibility is that while a T-jump in isometric conditions does not change the number of attached heads (Coupland *et al.* 2005 and references therein), a T-jump during shortening is accompanied by recruitment of new heads; at the same absolute shortening velocity, the number of attached heads would be greater at a higher temperature because V_0 is larger at higher temperature and hence the shortening after the T-jump occurs at a lower relative velocity. On the basis of similar temperature dependence (Figs 7 and 8), however, the T-jump-induced tension rise in shortening muscle seems to contain an endothermic step, similar to phase 2b in isometric muscle. Therefore, a clear finding here is that the endothermic force generation process is evident in isometric muscle, it is pronounced during steady muscle shortening, but it is depressed or inhibited, during muscle lengthening.

T-jumps during steady muscle shortening and lengthening

In principle, the active muscle force in an isometric contraction is maintained by an 'equilibrium' between low and high force, attached crossbridge states (actomyosin motor conformations), so that a small rapid length release (a shortening, inducing negative strain on attached crossbridges) will reduce force and perturb the equilibrium leading to a force generation (Huxley & Simmons, 1971). Conversely, a small rapid stretch (a lengthening, inducing positive strain on crossbridges) would also perturb the equilibrium but lead to an inhibition/reversal of the force generation. In other words, the actomyosin conformational change (the power stroke) leading to muscle force generation is more likely to occur when exposed to negative strain than when exposed to positive strain.

From the above considerations, and that a T-jump is thought to perturb an early molecular step so that the T-jump tension response can be used as a 'signature'

of the actomyosin ATPase cycle in active muscle, our findings naturally lead to the following important conclusion. The enhanced endothermic force generation, signalling the force-generating conformational change of the motor, suggests that the ATPase cycle proceeds more readily during steady muscle shortening – as found in studies on muscle energetics (see references in Woledge *et al.* 1985; Smith *et al.* 2005); indeed, the need to have a fast crossbridge cycling rate (a high detachment rate) during shortening was well recognized in crossbridge modelling (Huxley, 1957). Conversely, it appears that, during lengthening, the myosin motor fails to undergo the force-generating transition and, hence, the crossbridge/actomyosin ATPase cycle becomes short-circuited, before phosphate release

Figure 8. Temperature dependence of the T-jump tension response during shortening

Pooled data are from 4 fibres (as in Fig. 6B) in each of which the tension response to a 3°C T-jump, when shortening at the same velocity (range $0.06\text{--}0.09 L_0\text{ s}^{-1}$ – in different experiments), was examined at different initial temperatures; the initial temperature was increased and clamped by the Peltier modules fixed to the trough wall. Lower panel, the mean ($\pm$ S.E.M.) amplitude of T-jump tension rise is plotted as a percentage of the post-T-jump tension (left ordinate) against the reciprocal absolute temperature on the abscissa; the dashed curve through the points is fitted by eye. The relative amplitude decreases with increase of temperature. Upper panel, an Arrhenius plot; logarithm of the rate of tension rise (right ordinate) versus reciprocal absolute temperature. The open symbols represent the mean ($\pm$ S.E.M.) values for the rate of T-jump-induced tension rise during shortening and the continuous line is the calculated linear regression (between the individual log rates, $n = 36$, and $1/T$, $P < 0.001$) and the dotted lines denote its 95% confidence limits; the slope corresponds to a Q_{10} of 2.64. The filled symbols and the crosses, respectively, represent the fast (phase 2b) and the slow (phase 3) rates from T-jumps in the isometric state, from one of the experiment; the Q_{10} values are 3 (fast) and 1.6 (slow), similar to the previous findings (Ranatunga, 1996).

and before energy liberation. Overall, this would lead to the well-known Fenn effect (Fenn, 1924) that energy liberation in muscle is enhanced during shortening and depressed during lengthening (Infante *et al.* 1964; Curtin & Davies, 1973; Linari *et al.* 2003b). Furthermore, the implication is that the high force in lengthening muscle (see Fig. 4) arises from the pre-power stroke crossbridges attaching, getting strained by stretch and detaching without proceeding through the full ATPase cycle, a conclusion that is also generally consistent with previous studies (Lombardi & Piazzesi, 1990; Getz *et al.* 1998; Pinniger *et al.* 2006). It is also noteworthy that, whereas the role of muscle tendon in energy storage and its relevance and significance in animal locomotion are well recognized (Alexander, 1992), within-muscle energy storage during lengthening is probably not fully appreciated (Linari *et al.* 2003b). The non-endergonic nature of steady tension during lengthening and, although not characterized here, a more obvious thermal-expansion effect by a T-jump (see Figs 1 and 2) indicate that stretch of non-crossbridge elements within muscle may contribute to the force and energy storage in lengthening muscle (Edman & Tsuchiya, 1996; Pinniger *et al.* 2006).

From isotonic release experiments on intact rat muscle, at different temperatures, it was found that the maximum shortening velocity and the steady state force-shortening

velocity curve were very temperature sensitive such that the maximal mechanical power output increased markedly (> 20 -fold) in warming from 10 to 35°C (Ranatunga, 1984, 1998). The present findings that the steady tension in shortening muscle is particularly sensitive to a T-jump (Figs 2 and 3) and that the tension at a given shortening velocity increases with warming, in a manner similar to the isometric force (Fig. 7), were basically expected from those studies. Additionally, the fact that the lengthening muscle tension is insensitive to a T-jump is also consistent with the eccentric force measurements made in human muscle experiments at high and low temperatures (De Ruiter & De Haan, 2001) and is found in steady state, frog fibre experiments (Fig. 6A of Piazzesi *et al.* 2003).

T-jump tension response in shortening muscle

(i) During approach to steady state. When a standard T-jump was applied at different times during a ramp shortening, it appeared that the T-jump tension response becomes more pronounced as the shortening continues and tension during shortening approaches the steady state (Fig. 6A and B). However, when the underlying tension decrease due to shortening is removed, the T-jump response remains similar at different times; thus, examination of the difference tension traces (Fig. 6C)

Figure 9. Simulated tension responses to a standard T-jump, during steady shortening

Simulations carried out using Scheme 1, where the sum of the fractional occupancy of three states ($AM^*.ADP.P_i$, AM^*ADP and $AM^{**}.ADP$) is taken as force – see Methods (in the plots, it is normalized to the sum of occupancy of the three states in isometric case (i.e. P_0 at 10°C)). In each case, a shortening was simulated by increasing k_{+4} and, at steady state, a 5°C T-jump (re-setting k_{+1}) is induced at time zero (see Gutfreund & Ranatunga, 1999). A, simulated tension responses to T-jumps, at ~10°C; initial force is reduced by simulated shortening at different velocities (low to high). Note that a T-jump induces a faster tension rise at higher velocities, but the absolute amplitude of tension rise is similar (as in Figs 3B and 5). B, simulated T-jump tension responses, when shortening at the same velocity (constant k_{+4}) at different temperatures (as labelled). The pre- and post T-jump tension and the rate of tension rise to a T-jump are increased at the higher temperatures (as in Figs 7 and 8).

indicates that the tension response to a T-jump and to a ramp shortening may represent separate events. This finding, which deserves to be experimentally addressed in more detail in the future, would be consistent with the general thesis (see above) that a T-jump enhances an early step, the force generation step, in the crossbridge cycle whereas shortening enhances (post-stroke) crossbridge detachment, a step near the end of the cycle. Figure 5 shows that a T-jump induces a constant amplitude tension rise at various velocities. A basically similar observation was made in our previous studies where we examined the tension response to a standard T-jump when fibre tension was decreased with inorganic phosphate (Fig. 2A in Ranatunga, 1999) and increased with MgADP (Fig. 2A in Coupland *et al.* 2005). Taken together, these findings imply that endothermic force generation is an isolated step, it is not directly coupled to phosphate release or to ADP release and, as the present results show, that it is isolated from the shortening-induced crossbridge detachment step (ratchet).

(ii) Simulations. Using a minimal, five-step, crossbridge/AM-ATPase kinetic scheme (similar to Scheme 1) that included a two-step phosphate release, where step 1 (only) generates force and is temperature sensitive (endothermic), and a subsequent slow, two-step MgADP release, we could qualitatively simulate many of the temperature-dependent features of isometric force (see Coupland *et al.* 2005). Therefore, we attempted to use such kinetic modelling simply to see whether simulations would, at least, show some of the features during shortening; no detailed analyses were carried out, however, since appropriate simulation of the present findings would indeed require specific consideration being given to the strain-sensitivity of various steps in the crossbridge cycle.

Figure 9A shows simulated (see Methods for detail) tension responses to a small T-jump at $\sim 10^\circ\text{C}$ where pre-T-jump force is reduced to steady state, by simulated shortening (increased k_{+4}). The T-jump-induced tension rise is faster as the force is decreased by higher shortening velocities (traces from top to bottom), as found in the experiments (Fig. 3B); also, the absolute amplitude of the T-jump tension rise remains similar (Fig. 5). Figure 9B shows simulated T-jump tension responses at different temperatures when the system was simulated to be in steady shortening at one velocity; the traces show that pre-T-jump force, during shortening, is increased and the T-jump force rises faster, as the temperature is raised – as found experimentally (Figs 7 and 8), and similar to isometric force (Coupland *et al.* 2005). The model in Scheme 1 does not identify the detached states separately but, when [ATP] is high as in muscle, changes in occupancy of [AM] (in the scheme) would indicate changes in occupancy of detached states (M.ATP, M.ADP.P_i); on that basis and compared with the isometric case, the occupancy

of detached states increases when shortening is simulated. Thus, when force is reduced to $\sim 50\% P_0$ by shortening at $\sim 10^\circ\text{C}$, the summed occupancies of all AM.XX states except [AM] is decreased to $\sim 70\%$ of the isometric value. This is broadly consistent with the experimental finding from frog muscle fibres that stiffness at $V_{\max}$ would be $\sim 40\%$ of the isometric value (Julian & Morgan, 1981; Ford *et al.* 1985); due to filament compliance, however, the stiffness in shortening fibres would over-estimate the number of attached crossbridges (Goldman & Huxley, 1994). Thus, in principle, a linear kinetic scheme seems able to highlight the main trends in our present findings, which are limited to a low velocity range on either side of isometric point; incorporation of strain-sensitive features and other temperature-sensitive steps, etc. (Ferenczi *et al.* 2005) would provide a more quantitative description and it would be worthwhile to extend the experimental findings to velocities approaching $V_{\max}$ during shortening.

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