Increase in ATP consumption during shortening in skinned fibres from rabbit psoas muscle: effects of inorganic phosphate

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1. The influence of inorganic phosphate (P_i) on the relationship between ATP consumption and mechanical performance under isometric and dynamic conditions was investigated in chemically skinned single fibres or thin bundles from rabbit psoas muscle. Myofibrillar ATPase activity was measured photometrically by enzymatic coupling of the regeneration of ATP to the oxidation of NADH. NADH absorbance at 340 nm was determined inside a miniature (4 μl) measuring chamber.

2. ATP consumption due to isovelocity shortenings was measured in the range between 0·0625 and 1 L_0 s^{-1} (L_0: fibre length previous to shortening, corresponding to a sarcomere length of 2·64 μm), in solutions without added P_i and with 30 mM P_i. To get an estimate of the amount of ATP utilized *during* the shortening phase, quick releases of various amplitudes were also performed.

3. After quick releases, sufficiently large that force dropped to zero, extra ATP was hydrolysed which was largely independent of the amplitude of the release and of the period of unloaded shortening. This extra amount, above the isometric ATP turnover, corresponded to about 0·7 and 0·5 ATP molecules per myosin head at 0 and 30 mM P_i, respectively.

4. ATP turnover during the isovelocity shortenings was higher than isometric turnover and increased with increasing shortening velocity up to about 2·7 times the isometric value. At low and moderate velocities of shortening ($< 0{\cdot}5$ L_0 s^{-1}), P_i reduced ATP turnover during isovelocity shortening and isometric ATP turnover to a similar extent, i.e. a decrease to about 77 % between 0 and 30 mM added P_i.

5. The extra ATP turnover above the isometric value, resulting from isovelocity shortenings studied at different speeds, was proportional to the power output of the preparation, both in the absence and presence of added [P_i].

6. The effect of shortening velocity and [P_i] on energy turnover can be understood in a cross-bridge model that consists of a detached, a non- or low-force-producing, and a force-producing state. In this model, mass action of P_i influences the equilibrium between the force-producing and the non- or low-force-producing cross-bridges, and shortening enhances cross-bridge detachment from both attached states.

In contracting muscle, the free energy available from the ATP hydrolysis reaction drives the molecular interaction between myosin and actin, the cross-bridge cycle. Energy turnover by the contractile apparatus is coupled to the mechanical performance of the muscle through the influence of cross-bridge distortion on cross-bridge kinetics (e.g. Huxley, 1957; Eisenberg, Hill & Chen, 1980; Hibberd & Trentham, 1986; Pate & Cooke, 1989*a*). To obtain a full picture of cross-bridge kinetics, measurements are required of both mechanical parameters, which reflect the faster reactions in the cross-bridge cycle, and the rate of ATP (energy) turnover, which probe the slower, rate-limiting step(s).

In previous studies of energy turnover by means of heat measurements (e.g. Fenn, 1923, 1924; Hill, 1970; Barclay, Constable & Gibbs, 1993; Buschman, Elzinga & Woledge, 1995) and by biochemical determinations (e.g. Infante, Klaupiks & Davies, 1964; Crow & Kushmerick, 1982; Heglund & Cavagna, 1987) the total energy output was measured, which also included energy from sources other than the cross-bridges. In skinned skeletal muscle fibres, in which the sarcolemma has been made permeable, it is possible to measure exclusively the myofibrillar ATP turnover by means of an enzymatic coupling of the resynthesis of ATP to the oxidation of NADH, present in the bathing solution (Glyn & Sleep, 1985; Stienen,

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Roosemalen, Wilson & Elzinga, 1990). However, until now only mean ATPase activity could be measured under isometric conditions or during repetitive changes in length (Stienen, Papp & Elzinga, 1993; Potma, Stienen, Barends & Elzinga, 1994*a*). Although there is evidence suggesting that the energetic effects of restretches are relatively small, it was not possible to separate the energy turnover associated with the different phases of these protocols of repeated releases and restretches. The high signal-to-noise ratio achieved with our new measuring chamber (volume 4 μl) permitted us to resolve changes in ATP turnover resulting from single isovelocity shortenings.

The power stroke of the cross-bridge is associated with the release of inorganic phosphate, i.e. the transition from the AM.ADP.P_i to the AM.ADP state (Hibberd, Dantzig, Trentham & Goldman, 1985; AM, actomyosin), and the rate of this transition may depend on cross-bridge strain (Dantzig, Goldman, Millar, Lacktis & Homsher, 1992; Walker, Lu & Moss, 1992). Phosphate also influences contraction parameters such as force, stiffness, isometric ATPase activity and calcium sensitivity (Hibberd *et al.* 1985; Nosek, Fender & Godt, 1987; Kawai, Güth, Winnikes, Haist & Rüegg, 1987; Pate & Cooke, 1989*b*; Martyn & Gordon, 1992; Iwamoto, 1995; Potma, van Graas & Stienen, 1995), but not other, dynamic, properties like the maximum shortening velocity and the shape of the force–velocity relation (Cooke & Pate, 1985; Chase & Kushmerick, 1988). Moreover, the energetic consequences of the rise in intracellular concentration of P_i, as a result of the ATP hydrolysis reaction, are of interest with regard to the development of muscle fatigue (e.g. Westerblad, Lee, Lännergren & Allen, 1991). Therefore, in this study we investigated the influence of inorganic phospate on energy turnover during movement.

A preliminary account of part of this work has appeared elsewhere (Potma & Stienen, 1995).

METHODS

Preparation and solutions

Anaesthesia, methods for dissection and handling of the fibre preparations, composition of the solutions and equipment were as described previously (Potma *et al.* 1994*a*; Potma, van Graas & Stienen, 1994*b*) and are summarized below.

Adult New Zealand White rabbits (*ca* 3 kg), were anaesthetized by injection of 60 mg kg^{-1} sodium pentobarbitone or 9·6 mg kg^{-1} fluanisone and 0·3 mg kg^{-1} fentanyl citrate (Hypnorm; Janssen Pharmaceutica B.V., Beerse, Belgium) in the ear vein, and were exsanguinated via the carotid artery. Fibre bundles of about 2 mm in diameter were obtained from the psoas muscle, and were stored at −18 °C in a 50% (v/v) glycerol solution for up to 2 months (Goldman, Hibberd & Trentham, 1984). Single fibre segments or small bundles of several (up to about 10) fibre segments were separated from these bundles, and kept for 1 h in relaxing solution to which 1% (v/v) Triton X-100 was added, to disrupt remaining membranes and remove sarcoplasmic reticulum ATPase activity. Subsequently, the ends were crimped into aluminium T-clips. The length of the preparation between the clips ranged from 1·95 to 2·55 mm (at sarcomere length 2·64 μm).

During the measurements the preparations were incubated in relaxing and activating solutions with low (0 mM added) and high (30 mM added) P_i concentration. The composition of these solutions was calculated using the equilibrium constants of Fabiato (1981). The relaxing solution contained 5 mM EGTA and the activating solution 5 mM CaEGTA. In addition the solutions contained: 100 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (Bes), 1 mM free Mg^{2+}, 5 mM MgATP, 1·2 mM NADH, 10 mM phosphoenolpyruvate (PEP), 4 mg ml^{-1} pyruvate kinase (470 U mg^{-1}, Sigma), 0·24 mg ml^{-1} lactate dehydrogenase (710 U mg^{-1}, Sigma), 5 mM sodium azide, 0·2 mM P^1,P^5-diadenosine-5′-pentaphosphate and 10 μM oligomycin B. Ionic strength was adjusted to 200 mM by adding potassium propionate, and pH was set to 7·1 (at 15 °C) with potassium hydroxide. By adding Ca^{2+} from a concentrated $CaCl_2$ solution, it was verified that all activations were carried out at saturating calcium concentration. The temperature of the solutions was controlled at 15 ± 1 °C.

ATPase assay

ATPase activity of the skinned psoas fibres was measured by a coupled enzyme assay, as described previously (Glyn & Sleep, 1985; Stienen *et al.* 1990; Potma *et al.* 1994*a*,*b*). After hydrolysis of ATP into ADP and P_i inside the fibre preparation, the ADP formed is resynthesized to ATP inside the solution by an enzymatic coupling, which eventually results in the oxidation of NADH to NAD^+. This reaction sequence is catalysed by pyruvate kinase and lactate dehydrogenase. The breakdown of NADH was determined photometrically from the absorbance at 340 nm of near-UV light which passes through the solution, underneath the fibres. To minimize noise in the ATPase signal due to scattering particles, activating solutions were filtered (pore size, 0·45 μm; Nihon Millipore, Yonezawa, Japan).

The reactions involved in the ATPase assay are (Bergmeyer, 1965):

$$\text{ATP} \rightarrow \text{ADP} + \text{P}_i, \quad (1)$$

$$\text{PEP} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP} \quad (2)$$

and

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-lactate} + \text{NAD}^+. \quad (3)$$

The properties and limitations of this assay can be assessed as follows. Reaction (1) (ATP hydrolysis) takes place inside the fibre and, under isometric steady-state conditions, occurs at a velocity of 0·4 mM s^{-1} (see Results). The equilibrium of the pyruvate kinase reaction (reaction (2)) lies far towards pyruvate (apparent equilibrium constant (K_{app}) at pH 7·1, ~6 × 10^3; McQuate & Utter, 1959; Reynard, Hass, Jacobson & Boyer, 1961) and the formation of L-lactate (reaction (3)) is highly favoured. ATP depletion and ADP build-up will limit ATP hydrolysis (reaction (1)) with a K_m of about 20 μM (Glyn & Sleep, 1985) and a K_i of 0·2 mM (Cooke & Pate, 1985; Sleep & Glyn, 1986), respectively. Hence reaction (2) (ATP resynthesis) should take place rapidly, inside the fibre. The speed of this reaction is determined by its maximum speed, V_{max}, which depends on the pyruvate kinase activity, its K_m for ADP and its K_i for pyruvate.

The activity of pyruvate kinase (PK; Sigma) used amounts to 470 U (mg protein)$^{-1}$ at 25 °C. Assuming that the activity at 15 °C (the temperature at which the experiments were carried out) is a factor of 3 lower, the activity of PK in solution (4 mg ml^{-1}) amounts to a V_{max} of ~10 mM s^{-1}. The activity of lactate dehydrogenase (LDH) is not very temperature sensitive around 20 °C (Biochemica Information, Boehringer Mannheim, Germany) and 0·24 mg ml^{-1} of LDH (710 U (mg protein)$^{-1}$) corresponds to a V_{max} of reaction (3) of 0·9 mM s^{-1}. The influence of the concentration of substrates ([S])

and products on the velocity of reactions can be estimated on the basis of published (apparent) K_m and K_i values (e.g. Biochemica Information and references therein), respectively, using the classic Michaelis–Menten equation:

$$V = V_{max} \times [S]/(K_m + [S]).$$

Since the MgATP concentration in the fibre (5 mM) is much larger than the K_m of reaction (1) (20 μM), ADP (product) inhibition on reaction (1) is neglibible (cf. Cooke & Pate, 1985). The K_m of ADP in the PK reaction amounts to 0·3 mM. Hence if the measured velocity of ATP consumption (0·4 mM s^{-1}) were limited by ADP, the ADP concentration would be 12 μM. This indicates that the (mean, free) ADP concentration in the preparation may rise to values around 10–50 μM. These are similar to the *in vivo* value (Veech, Lawson, Cornell & Krebs, 1979). The K_m for pyruvate in reaction (3) amounts to 0·12–0·15 mM. If the measured velocity of ATP consumption were limited by this K_m, the pyruvate concentration would be ~0·12 mM. The speed of reaction (2) is only slightly inhibited at pyruvate concentrations of 2 mM. Therefore it is unlikely that the pyruvate concentration could rise to a level where it would have an inhibiting effect on the velocity of ATP resynthesis. From these calculations is seems plausible that the assay is dimensioned sufficiently for an adequate measurement of the steady-state velocity of ATP consumption. During short bursts of ATP hydrolysis, lasting for about 1 s, which may occur during shortening, transient concentration changes in the substances involved could take place but even for the maximum velocity observed ~1 mM s^{-1} (see Results), none of the substances formed or used could become rate limiting. Reaction (3) might be the most critical in this aspect. It was found, however, that the effect of a 5-fold increase in the LDH concentration on isometric ATP consumption as well as on the time course and the magnitude of the change in ATP consumption as a result of isovelocity shortening was negligibly small.

One comment should be made on the PEP concentration. In previous experiments, in which rather thick fibres of the iliofibularis muscle of *Xenopus laevis* were studied at 20 °C (Stienen, Zaremba & Elzinga, 1995), it was noticed that a PEP concentration of 5 mM was critical for the ATPase measurements and therefore we decided to use a PEP concentration of 10 mM in the bathing solutions of the present study. A critical PEP concentration in these previous experiments would be compatible with a diffusion constant for PEP inside the preparation of about 300 μm^2 s^{-1}, which is similar to the published value for inorganic phosphate (Yoshizaki, Seo, Nishikawa & Morimoto, 1982). Using this diffusion constant, it can be calculated that the minimum PEP concentration during isometric contraction, in the core of a cylindrical bundle of psoas fibres of 200 μm diameter, would be ~6·7 mM. Even without any diffusion, this concentration of PEP would be sufficient for a burst of activity at a velocity of ~1 mM s^{-1} lasting several seconds. The critical preparation diameter (calculated for an ATPase activity corresponding to 0·4 mM s^{-1}, a PEP concentration of 10 mM and the diffusion constant given above) is 340 μm. The thickest fibre bundle used in this study had a cross-sectional area of 36 000 μm^2 (mean diameter, 220 μm).

Experimental set-up and protocol

The fibre preparation was mounted in the apparatus by means of T-clips (Fig. 1) between a force transducer (AE801, Sensonor, Horten, Norway) and a displacement generator (Model 101, Ling Dynamic Systems, Royston, UK). The latter was controlled by a Z-80-based programmable function generator.

The preparation could be transferred manually between two anodized aluminium troughs (volume 80 μl) and a 4 μl chamber, in which the ATPase activity was measured. One of the 80 μl troughs contained relaxing solution, the other one activating solution. In this latter trough the preparations were activated before they were transferred into the measuring chamber, to prevent the fibres touching the sides of this very narrow chamber (dimensions: length, 3 mm; width, 0·4 mm; depth, 3 mm). To ensure a constant optical path length inside the solution, small windows covered the lower 1·5 mm of the chamber, through which the UV light passed. Surface tension kept the solution inside the upper half of the chamber, in which the preparation could be immersed. During measurement the preparations were fully immersed in solution, but the clips were kept partially outside. Three syringes could be inserted via separate inlets. With the first syringe, the inlet of which could be closed by a valve, the solution could be replenished from the bottom of the chamber. The second syringe was used to stir the solution, by injecting and aspirating 0·4 μl at a rate of 15 s^{-1}. The third injector was controlled by a stepper-motor and was used to add 0·005 μl of 10 mM ADP at the end of each recording, to calibrate the absorbance signal. The data were recorded on a chart recorder and on a computer at a sampling rate of 20 Hz. Force and length were also sampled at a faster rate of 1 kHz for 15 s, starting 1 s before shortening.

From each recording, values were obtained for the steady-state isometric ATPase activity before the preparation was shortened (at L_0, corresponding to sarcomere length 2·64 μm) and after shortening (at a shorter length, L_e), as illustrated in Fig. 2. The extra amount of ATP utilized associated with the shortening was determined too. Isometric ATPase activity was derived by linear regression analysis of the absorbance signal. Force and absorbance signals measured in relaxing solution (pCa 9) served as a baseline for the active force and ATPase activity levels. Previously we found that neither $[P_i]$ of the relaxing solution nor length changes influenced basal ATPase activity (Potma *et al.* 1994*a*, 1995).

Before the first activation–relaxation cycle, sarcomere length, measured in relaxing solution by means of helium–neon laser diffraction, was adjusted to 2·64 μm. During the first contracture the preparation was kept isometric. After that, if necessary, sarcomere length was readjusted to 2·64 μm, the motor displacement signal was programmed, and the length of the preparation between the clips as well as its width and depth were measured. The experiment started with the motor switched off. The preparation was activated and then transferred into the measuring chamber. After stabilization, isometric ATPase activity (A_0) and force (F_0) at L_0 were measured. Subsequently the motor was switched on and the preparation was shortened, either at a constant velocity or by a quick (2 ms) release. After ATPase activity and force became stable again, their isometric values at the final length (A_e and F_e at L_e) were measured. If A_0 and A_e were equal, the amount of ATP hydrolysed that was associated with the shortening, ΔATP, was the offset between the regression lines corresponding to A_0 and A_e (cf. Fig. 2). However, although the mean ratio of A_e/A_0 was not significantly different from 1, A_0 and A_e were not always identical. To find ΔATP in such a case, the regression line with the higher slope was substracted from the ATPase signal. For $A_0 > A_e$, in the resulting difference signal ΔATP was taken as the difference between the horizontal line corresponding to A_0 and the minimum of the rest of the signal. This minimum corresponds to the end of the increased rate associated with shortening. For $A_e < A_0$, ΔATP was taken as the difference between the maximum of the signal (corresponding to where the increased ATP turnover rate begins) and the horizontal line (corresponding to A_e). It should be noted that this method always gives a slight underestimate of ΔATP. After the preparation

was returned to the relaxing solution, it was restretched to L_0. Thereafter the absorbance baseline – a result of NADH bleaching under the intense UV light, contaminating ATPases present in the enzymes used and evaporation of the solution – was determined and a 50 pmol ADP calibration injection was given. For each preparation a series of these activation–relaxation cycles was performed until F_0 became less than 80% of its value during the first activation.

For the isovelocity shortenings (ΔL) an amplitude of 20% of L_0 was chosen. The shortening velocity, and therefore the duration of the shortening ramp (Δt), was varied between 0·0625 L_0 s^{-1} ($\Delta t = 3{\cdot}2$ s) and 1 L_0 s^{-1} ($\Delta t = 200$ ms). For the quick releases, which started from L_0 and were performed in 2 ms, four different amplitudes were used with a ΔL of 5, 10, 15 or 20% of L_0. The effects of shortening velocity or amplitude were tested in random order. To minimize expensive rinsing of the solution inside the measuring chamber, experiments on one preparation were performed in most cases at either 0 or 30 mM P_i. For each activation all measured values were normalized on the isometric values obtained before the shortening (A_0, F_0). A mean value was calculated from these normalized values. In the figures, mean ΔATP and ATPase activity are shown calculated from the normalized values of each experiment multiplied by the mean A_0 of all the preparations. The ΔATP and ATPase values were expressed in molecules per myosin head, assuming a mean myosin head density of 0·2 mM (e.g. Glyn & Sleep, 1985).

To get an estimate of the ATPase activity *during* the shortening phase, the absorbance change corresponding to ΔATP was corrected for NADH breakdown that was not directly associated with the shortening phase, on the basis of the results of the quick release experiments. The mean rate of ATP consumption thus obtained can be compared with the mean rate of work produced, i.e. the mean power output of the preparation. The mean power output of a preparation shortening at constant velocity was calculated from the mean force during the shortening period multiplied by shortening velocity.

Values are given as means ± S.E.M., except where noted otherwise. All statistical statements are based on Student's two-tailed t tests ($P < 0{\cdot}05$).

RESULTS

The mean isometric ATPase activity per preparation volume (A_0) and the isometric force level per cross-sectional area (F_0) were measured for the second contraction of each preparation at a sarcomere length of 2·64 μm. At 0 P_i, A_0 and F_0 values were 0·42 ± 0·03 mM s^{-1} and 141 ± 6 kN m^{-2}, respectively ($n = 16$), and at 30 mM P_i, values were 0·32 ± 0·02 mM s^{-1} and 79 ± 4 kN m^{-2}, respectively ($n = 11$).

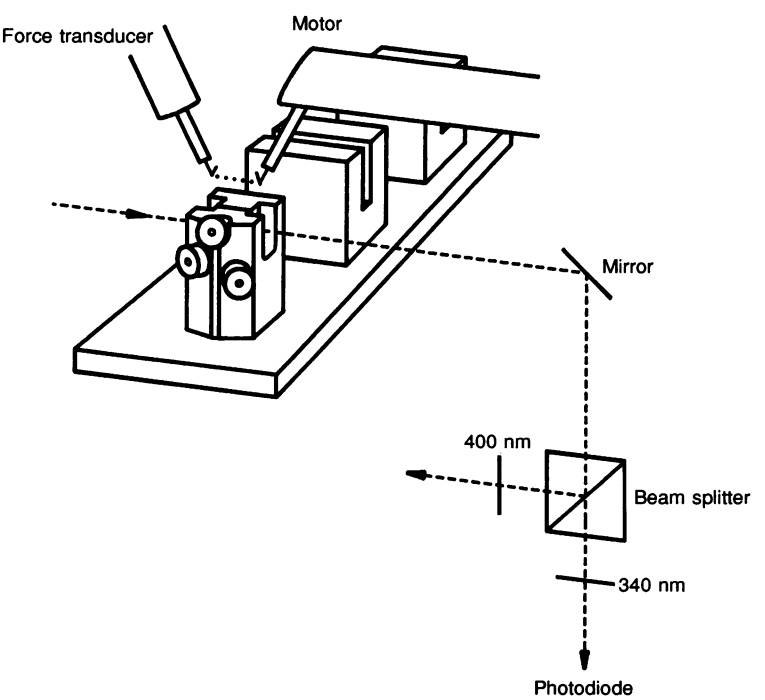

Figure 1. Diagram of the experimental set-up

The preparation (dotted line) was attached to a motor arm and a force transducer and could be transferred between 2 troughs (volume, 80 μl) filled with relaxing and activating solution, respectively, and the measuring chamber (volume, 4 μl; nearest in the figure). The NADH absorbance, which was enzymatically coupled to ATP hydrolysis, was derived from the light intensity at 340 nm measured by a UV-enhanced photodiode, after a 50/50 beam splitter. The light intensity at 400 nm was used to correct the absorbance signal for intensity variations, due to variation of the incident light or to scattering particles in the optical path. Through the three inlets shown, syringes can be inserted into the measuring chamber for replenishment of the solution, for stirring, and for ADP injection (calibration).

In Fig. 2, registrations of length, force and NADH absorbance can be seen, for a fibre bundle shortening at a constant velocity of 0·25 L_0 s^{-1}. For clarity signals are only shown for a 6 s period during which the preparation was activated and inside the measuring chamber. Examples of full recordings without length changes, which show activation, relaxation and ADP injection, can be found in Potma *et al.* (1994*b*). After activation the preparation was transferred into the measuring chamber in which it was contracting isometrically at L_0, the last second of which is shown in Fig. 2. Then a 20% isovelocity release was given, lasting 800 ms after which the fibres were kept isometric again at L_e. After a rapid drop, force declined at a fairly constant rate during the shortening, reflecting that in skinned fibres isovelocity shortenings are not always isotonic (e.g. Brenner, 1986). After the release force recovered towards the isometric level at the shorter length, F_e. Initially the slope of the absorbance signal was constant, corresponding to the isometric ATPase activity A_0. As a result of the release NADH was temporarily consumed at a faster rate, but the slope of the signal returned to a constant value, giving the isometric A_e, which in this example was very similar to A_0. Despite vigorous stirring, the absorbance signal was delayed by about 1 s with respect to length and force signals because of the mixing characteristic of the chamber.

In Fig. 2 an example is shown in which A_0 and A_e were equal, and ΔATP is the offset between the two isometric (dashed) lines. The mean of the ratio A_e/A_0, obtained for each experiment, amounted to $0{\cdot}97 \pm 0{\cdot}02$ ($n = 97$). This indicates that A_0 and A_e were not significantly different (cf. Stephenson, Stewart & Wilson, 1989, who found in skinned extensor digitorum longus (EDL) fibres from rat that isometric ATPase activity was constant at sarcomere lengths between 1·4 and 2·7 μm). This was the case both without and with added phosphate. Isometric force, however, was decreased at the lower length, as can be seen in Fig. 2. The ratio F_e/F_0 proved to be independent of shortening velocity and of $[P_i]$ and had a mean value of $0{\cdot}63 \pm 0{\cdot}01$ ($n = 97$).

In Fig. 3, experimental recordings of length, force and NADH absorbance are shown for a fibre bundle subjected to a quick release with an amplitude of 20% of L_0, in a solution without added phosphate. Analogous to Fig. 2, 1 s of isometric contraction at L_0 is shown. Then the 2 ms release was given, after which the fibre bundle was kept isometric at L_e. During the release, force dropped to zero. The bundle remained slack for a short period, not visible on the time scale of Fig. 3, and then force redeveloped towards the isometric end level F_e. The absorbance signal again shows very similar isometric ATPase values preceding and following the release, and an additional ΔATP associated with the release.

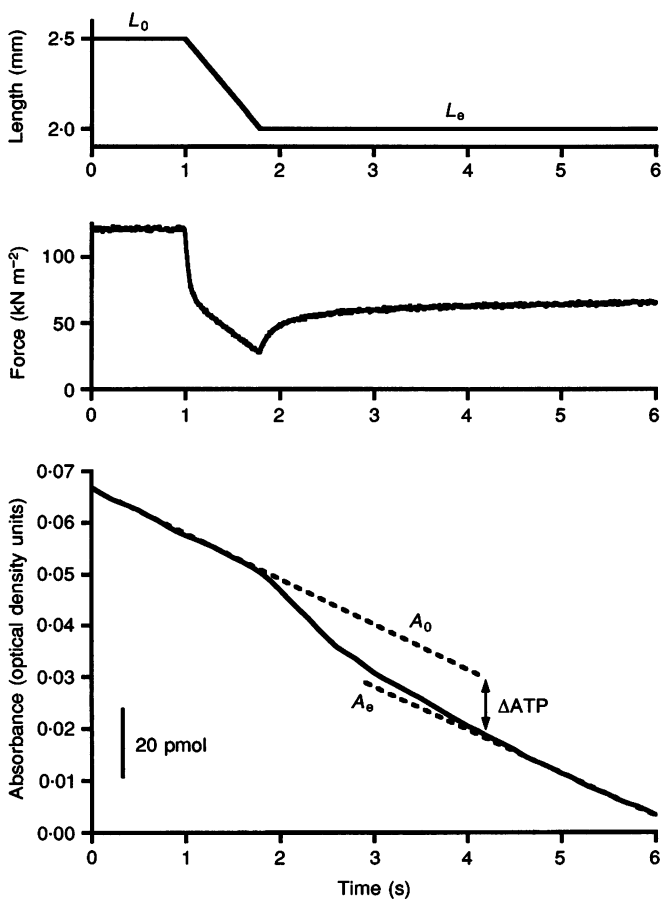

Figure 2. Increase in ATP consumption with isovelocity shortening

Recordings of length (upper trace), force (middle trace) and ATPase activity (bottom trace) for an isovelocity release. A bundle of 4 psoas fibres, maximally activated (pCa 4·5, 0 P_i added, pH 7·1), was contracting isometrically (at L_0, corresponding to a sarcomere length of 2·64 μm) during the first second of the recording shown. Then it was shortened by 20% of its length, at a constant velocity of 0·25 L_0 s^{-1}. Subsequently it was kept isometric again at the shorter length, L_e. High sample rate (1 kHz) recordings of the length and force signals are shown. NADH absorbance was sampled at 20 Hz. The slopes of the absorbance change (in optical density units; zero level arbitrarily chosen) are a measure of the rate of ATP hydrolysis (dashed lines: isometric ATPase activities at the initial length, A_0 and at the final length, A_e, both ~0·38 mM s^{-1}). During shortening this rate is increased. Due to the mixing characteristic of the chamber (time constant, ~1 s) the change in absorbance follows the other tracings with some delay. ΔATP is the extra amount of ATP hydrolysed resulting from the shortening (here 18 pmol). Dimensions of the fibre bundle (at sarcomere length 2·64 μm): length, 2·50 mm; and diameter measured from two perpendicular directions, 140/170 μm.

For the quick releases the ratio A_e/A_0 was not significantly different from 1 and independent of both ΔL and $[P_i]$. As could be expected, F_e/F_0 decreased with the amplitude of shortening ΔL. However a different decrease was found for low and high phosphate concentrations; for ΔL values of 5 and 10% of L_0, F_e/F_0 was significantly higher in 30 mM P_i than in 0 P_i (see Table 1).

The force records showed a faster redevelopment following the slack period in 30 mM P_i compared with 0 P_i. So for 0 P_i force redevelopment may not have always been complete, which could explain the lower mean 'isometric' final force in 0 P_i. Much longer activations were not feasible, because NADH would become depleted for the relatively thick bundles which had to be used for the quick releases to achieve sufficient precision in ΔATP. However, as can be seen in Figs 2 and 3, the slope of the NADH signal stabilized long before F_e was completely reached.

In Fig. 4 both the duration of the slack period and the additional amount of ATP hydrolysed, ΔATP, are shown as a function of the amplitude of the quick release, ΔL. All preparations showed a slack period except ΔL of 5% L_0 and 0 P_i. In this case, minimum force reached immediately after the release had a mean value of $0{\cdot}10 \pm 0{\cdot}01$ F_0. For the higher amplitudes, and at 30 mM P_i for all amplitudes, the duration of the slack period increased linearly with the amplitude of the quick release. From the slopes of the linear regression lines shown in the figures the maximum shortening velocities (± standard error of the estimate) were calculated to be $5{\cdot}0 \pm 0{\cdot}5$ and $4{\cdot}7 \pm 0{\cdot}2$ L_0 s^{-1} for 0 and 30 mM P_i, respectively. For 0 P_i no influence of the duration of the slack period on ΔATP can be seen, for the highest three amplitudes ΔATP was not significantly different. Only for $\Delta L = 5\%$, where the preparation did not go slack, ΔATP is smaller. At 30 mM P_i, the situation seems to be different.

Table 1. Ratio of the isometric forces which follow and precede the quick releases

ΔL (% of L_0)	F_e/F_0 0 mM P_i	F_e/F_0 30 mM P_i
5	$0{\cdot}88 \pm 0{\cdot}01$ (8)	$0{\cdot}95 \pm 0{\cdot}01$ (8)
10	$0{\cdot}80 \pm 0{\cdot}02$ (8)	$0{\cdot}91 \pm 0{\cdot}01$ (9)
15	$0{\cdot}81 \pm 0{\cdot}04$ (9)	$0{\cdot}86 \pm 0{\cdot}03$ (9)
20	$0{\cdot}67 \pm 0{\cdot}05$ (5)	$0{\cdot}73 \pm 0{\cdot}04$ (7)

Results are given as means ± S.E.M. with number of records given in parentheses.

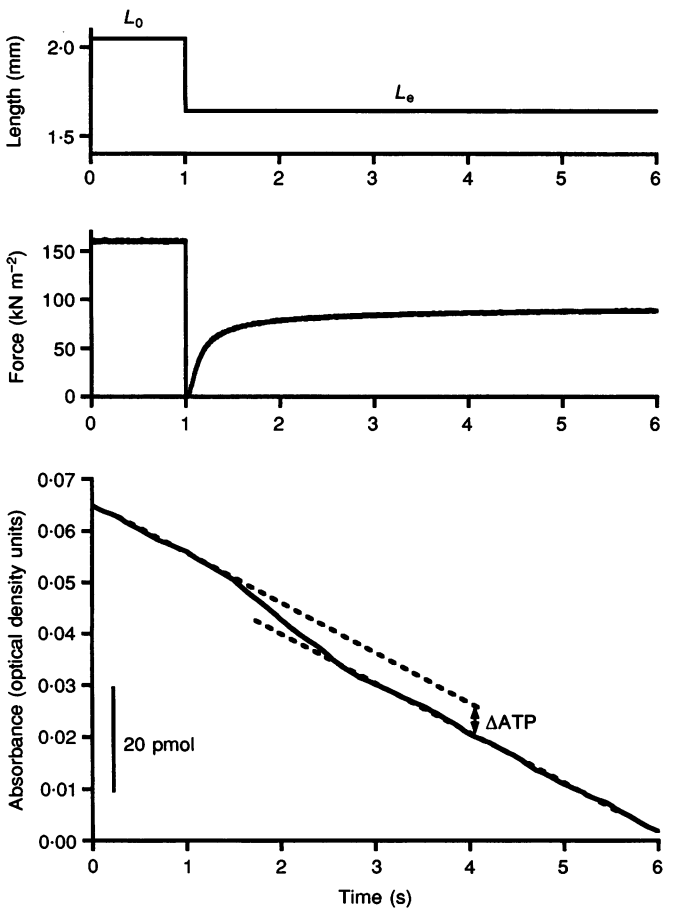

Figure 3. Increase in ATP consumption with a quick release

Recordings of length (upper trace), force (middle trace) and ATPase activity (bottom trace) for a quick release. After contracting isometrically at L_0 during the first second of the recording shown, a bundle of 6 fibres (0 P_i added, pH 7·1), was shortened by 20% of its length in 2 ms, and subsequently kept isometric again at L_e. After the release force remained zero for 23 ms (not visible on this time scale) during which the preparation shortened at maximum velocity. The dashed lines in the bottom panel (NADH absorbance) correspond to the isometric ATPase activity of 0·43 mM s^{-1}. As a result of the quick release an extra amount of ATP is hydrolysed, ΔATP (in this case 6 pmol). Its effect on the absorbance tracing is delayed by the mixing characteristic of the chamber. Dimensions of the fibre bundle (at sarcomere length of 2·64 μm): length, 2·05 mm and diameter measured from two perpendicular directions, 120/260 μm.

As ΔL increased, a gradual increase in ΔATP was observed. Nevertheless in both 0 and 30 mM P_i most of ΔATP was independent of ΔL, and thus of the time during which the fibres were shortening at maximum shortening velocity. Apparently a substantial additional amount of ATP was hydrolysed (or ADP released), above the isometric ATPase activity, which was not directly associated with the phase during which the preparation was shortening. This shortening phase-independent amount, ΔATP_i, was estimated to be about 0·7 and 0·5 ATP molecules per myosin head at 0 and 30 mM P_i, respectively (cf. Fig. 4 and Discussion).

For the isovelocity shortenings in almost all cases the preparation did not go slack (cf. Fig. 2). F_{min}, the minimum force reached at the end of the shortening, decreased monotonically with increasing shortening velocity. Between 0·0625 and 1 L_0 s^{-1}, F_{min}/F_0 decreased from 0·37 ± 0·03 ($n = 5$) to 0·02 ± 0·01 ($n = 7$) in 0 P_i and from 0·37 ± 0·04 ($n = 5$) to 0·12 ± 0·01 ($n = 7$) in 30 mM P_i. As a result of this, the mean force during the shortening phase, $\bar{F}$, decreased more at high shortening velocities in 0 P_i than in 30 mM P_i. Between 0·0625 and 1 L_0 s^{-1} force, $\bar{F}/F_0$ decreased from 0·56 ± 0·02 ($n = 5$) to 0·15 ± 0·02 ($n = 7$) at 0 P_i, and from 0·53 ± 0·02 ($n = 5$) to 0·24 ± 0·01 ($n = 7$) at 30 mM P_i. At 0·5 and 1 L_0 s^{-1}, $\bar{F}/F_0$ was significantly lower in 0 P_i than in 30 mM P_i. For an isovelocity shortening, the mean power output is equal to the product of $\bar{F}$ and the shortening velocity, and therefore the fact that $\bar{F}$ is decreased less at high velocities in 30 mM P_i than in 0 P_i translates into relatively high power outputs at high velocities in 30 mM P_i, despite of the marked reduction in isometric force by P_i.

In Fig. 5 the mean power output and ATP turnover rate are shown as a function of shortening velocity for 0 and 30 mM P_i. The steeper increase of power output at high velocities in

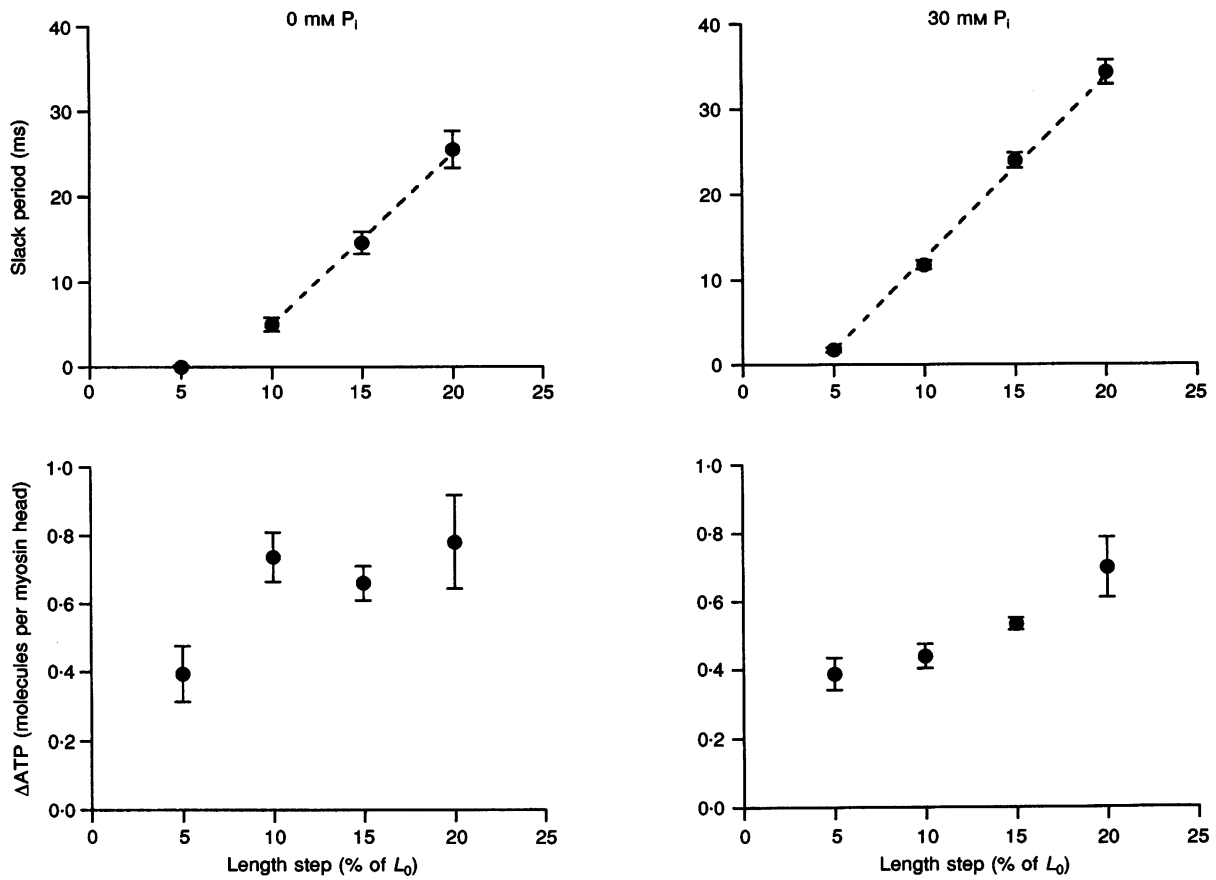

Figure 4. Extra amount of ATP consumed with quick releases of different amplitudes

Period during which the fibres remained slack (upper panels) and the extra ATP hydrolysed (lower panels) after quick (2 ms) releases of different amplitudes, without added P_i (left panels) and with 30 mM P_i (right panels). For different amplitudes of length step (ΔL = 5–20% of L_0), the fibre was shortening at maximum shortening velocity (~5 L_0 s^{-1}, from the linear regression lines in the upper panels) during the slack period, and subsequently isometric force redeveloped. Most of ΔATP, the extra amount of ATP hydrolysed above the isometric ATPase activity, appears to be independent of the slack period and therefore almost all of it is associated with the early rapid force reduction or with force recovery after the slack period. A mean myosin head density of 0·2 mM was assumed. Data points represent means ± S.E.M. ($n \geq 5$).

30 mM P_i can be seen in the upper panels. Since the total length change was always 20% of L_0, the duration of the shortening and of the ATP turnover associated with that phase decreased with increasing shortening velocity. The mean ATP turnover rate for the shortening, which is shown as the filled circles in the lower panels, was obtained for each velocity by dividing mean ΔATP by duration of shortening, and adding the result to the mean isometric ATP turnover rate $\bar{A}_0$. The strong similarity in shape in the upper and lower panels of Fig. 5 indicates that the additional ATP turnover, above the isometric rate, was proportional to the power output of the preparation. The results in the absence

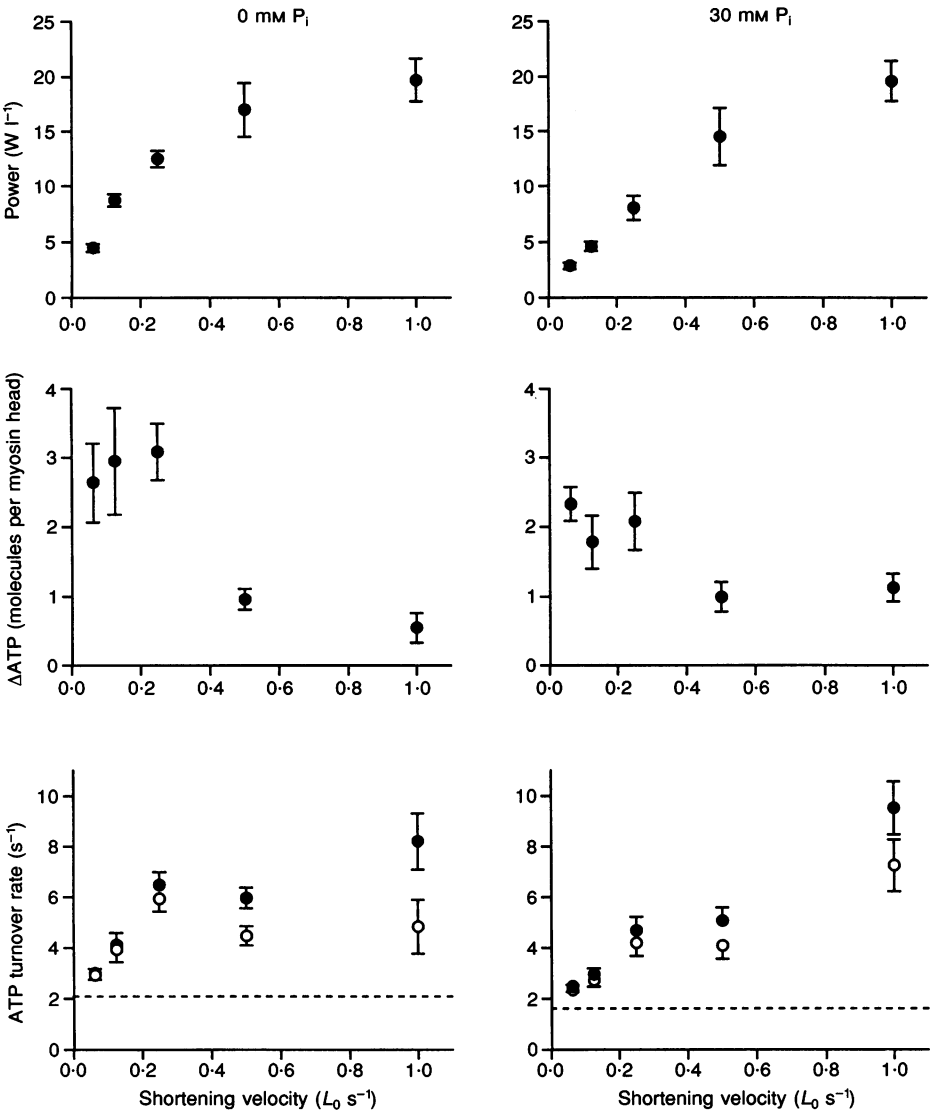

Figure 5. Relation between power output and ATP consumption

The mean power output expressed per preparation volume (upper panels), the extra ATP hydrolysed (ΔATP) as a result of shortening (middle panels) and the ATP turnover rate (lower panels), at different shortening velocities, without added P_i (left panels) and with 30 mM P_i (right panels). The mean power delivered by the shortening preparation (upper panels) was the product of the mean force during the shortening phase and the shortening velocity. To obtain the total ATP turnover rate associated with the shortening, which is shown as the filled circles in the lower panels, the uncorrected ΔATP was divided by the duration of the shortening and added to the isometric turnover. The latter is represented by the dashed line. A measure of the ATP turnover rate during the shortening is shown as the open circles in the lower panels. To obtain this measure the extra ATP hydrolysed, ΔATP, was corrected for the extra amount of ATP not directly associated with the isovelocity phase (on the basis of the results shown in Fig. 4). The ΔATP values that result after this correction are shown in the middle panels. Subsequently the corrected ΔATP was divided by the duration of the shortening and added to the isometric turnover. Note that the total length change was always 20% of L_0, so the durations of the releases were different. Data points represent means ± S.E.M. ($n \geqslant 5$).

and presence of added phosphate can be compared by normalizing the ATP turnover on A_0 (which was reduced to 77 ± 6% between 0 and 30 mM P_i) and the power output on F_0 (reduced to 56 ± 4% between 0 and 30 mM P_i). Between 0 and 30 mM P_i, these normalized values of the ATP turnover rate were identical except for 1 L_0 s^{-1}. The normalized values of the power output differed significantly between 0 and 30 mM P_i at 0·5 and 1 L_0 s^{-1}.

To get an estimate of the ATPase activity during the shortening phase, for each isovelocity measurement a correction ΔATP_{corr} was subtracted from ΔATP, which was obtained according to:

$$\Delta ATP_{corr}/A_0 = (\Delta ATP_i/\bar{A}_0)(F_e - F_{min})/F_e,$$

in which ΔATP_{corr} equals the amount of ATP utilized that was not directly associated with the isovelocity shortening phase. ΔATP_i was the mean extra amount of NADH breakdown, independent of the shortening phase, found for the quick release experiments, and $\bar{A}_0$ was the mean A_0 for all measurements. Using this approach, ΔATP_i was weighed by the fraction of force which had to be recovered. It should be noted that A_0, F_e and F_{min} were obtained from each individual recording, whereas ΔATP_i and $\bar{A}_0$ represent mean values. A validation of this procedure will be given in the Discussion. For each velocity the mean corrected ΔATP, which is shown in the middle panels of Fig. 5, was divided by the duration of the shortening and the result was added to the mean isometric ATP turnover rate A_0. The rate of ATP turnover directly associated with the shortening phase thus obtained, is shown as open circles in the lower panels of Fig. 5.

At the three lower velocities no significant change in the corrected ΔATP was observed, both in the absence and presence of added phosphate. Therefore the ATP turnover rate increased linearly with increasing shortening velocity up to 2·8 ± 0·2 A_0 in 0 P_i and to 2·6 ± 0·3 A_0 in 30 mM P_i ($n = 19$ in both cases). At higher velocities, the turnover rate seemed to stabilize or to decrease. It should be noted that the ATPase activity calculated for 1 L_0 s^{-1} is not only subject to a relatively large uncertainty due to the short duration of the release, but that its value is also very sensitive to the estimate of ΔATP_{corr}. For 1 L_0 s^{-1} the magnitude of ΔATP_{corr} was similar to that of ΔATP associated with the shortening phase. The relative increase in ATPase activity during the shortening with respect to the isometric ATPase differed between 0 and 30 mM P_i only at 1 L_0 s^{-1}. For the other velocities, the ATPase activity during shortening differed between 0 and 30 mM P_i by the same factor which was found for the isometric turnover.

DISCUSSION

Comparison with previous studies

The mean isometric ATPase activity (2·1 ± 0·1 per myosin head per second; assuming a myosin head concentration of 0·2 mM, e.g. Glyn & Sleep, 1985) and the mean isometric force (141 ± 6 kN m^{-2}) found in this study for maximally activated psoas fibres in low phosphate solution, correspond well with previous results (e.g. Glyn & Sleep, 1985; Kawai *et al.* 1987; Cooke, Franks, Luciani & Pate, 1988; Potma *et al.* 1994*a*, *b*, 1995). After normalization of the volume and cross-sectional area of the preparation, mean values of isometric ATPase and force at low and high [P_i] can be compared. At 30 mM P_i, A_0 and F_0 were reduced to 77 ± 6 and 56 ± 4%, respectively ($n = 11$), of their values at 0 P_i ($n = 16$). In our previous study on single fibre segments which were contracting isometrically at sarcomere length 2·4 μm (Potma *et al.* 1995), it was found that ATPase and force in psoas were depressed by 79 ± 3% and 48 ± 2% ($n = 9$). The lower P_i inhibition of force found in this study can be explained by a relatively high [P_i] inside the fibres in the solution with zero added P_i, because in the multifibre preparations P_i accumulation due to ATP hydrolysis (which is proportional to the square of the diameter of the preparation) is higher than in single fibres.

The increases of ATPase activity observed in this study and during comparable protocols of repetitive changes in length, in skinned rabbit psoas fibres (Arata, Mukohata & Tonomura, 1979; Kawai *et al.* 1987; Potma *et al.* 1994*a*), in insect flight muscle (Güth, Poole, Maughan & Kuhn, 1987) and in rat cardiac trabeculae (Ebus, Stienen & Elzinga, 1994) are very similar. The mean force during the single isovelocity shortenings in this study was slightly lower than the mean force during similar shortenings alternated with fast restretches, and the increase in ATPase during the single isovelocity shortenings was larger than the increase in ATPase during the repetitive protocol (Potma *et al.* 1994*a*). Part of this difference is due to the fact that in the earlier study some non-myofibrillar ATPase activity was measured because the fibres were not treated with Triton X-100. Moreover, although for the ramps/restretches the energetic effect of the shortening ramps prevails, ATP splitting may be reduced during the restretches (cf. Infante *et al.* 1964).

A potential artifact in the experiments, related to pyruvate accumulation inside the preparation, needs to be considered. It has been observed in skinned fibres that the distance between the actin and myosin filaments does not change greatly with sarcomere length (Matsubara & Elliot, 1972). Therefore the actual volume occupied by the fibre will decrease during shortening. If pyruvate accumulates inside the preparation, e.g. because pyruvate production (reaction (2) of the ATPase assay) would be faster than its breakdown (reaction (3)) and because of limitations in diffusion, pyruvate would be injected into the bathing solution. This would give rise to a transient acceleration of NADH breakdown. This would be followed, after the release, by a transient deceleration, since the final isometric ATPase activity was very similar to the initial isometric steady-state ATPase activity. Assuming a diffusion constant for pyruvate of 300 μm^2 s^{-1} and a preparation diameter typically between 100 and 200 μm, it can be calculated (cf. Stienen *et al.* 1990)

that this equilibration would occur with a half-time of 0·5–2 s. Since the extra ATP consumed was derived from the offset between the slopes representing initial and final ATPase activity and because pyruvate consumption takes place rapidly, we consider the influence of this potential artifact negligible.

The effect of $[P_i]$ on the ATP turnover during single shortenings can be compared with the results of Ebus *et al.* (1994) and Kawai *et al.* (1987). Kawai *et al.* (1987) used sinusoidal oscillations with relatively small amplitudes (0·5–1·5% of fibre length) and likewise found small increases in the ATP turnover rate (~6% at 100 Hz). These relative increases were independent of $[P_i]$. Ebus *et al.* (1994) found that the effect of P_i on ATPase activity during square-wave-shaped length changes was very similar to the effect on isometric ATPase activity. Thus the findings of both studies are consistent with our observation that, at 0·5 L_0 s^{-1} and below, P_i affects the ATP turnover under dynamic conditions and the isometric ATP turnover by the same factor.

The effects of phosphate on mechanics which were found here are also in good agreement with earlier studies. The enhancing effect of P_i on the speed of force recovery has been observed before (Hibberd *et al.* 1985, Ebus *et al.* 1994). Cooke & Pate (1985), Chase & Kushmerick (1988) and Pate & Cooke (1989*b*) also found that maximum shortening velocity was not sensitive to changes in $[P_i]$. In load clamp experiments on rabbit psoas (10 °C), Cooke & Pate (1985) found that 12 mM P_i did not affect the force–velocity relation.

ATP turnover not associated with the shortening phase

The amplitude-independent amount of extra ATP consumption, ΔATP_i, found for the quick releases, is probably associated with either the early rapid drop in force or with force recovery. Cross-bridges, during isometric contraction attached in the ADP-bound force-producing state, AM.ADP, may be forcibly detached by a release. Subsequently ADP would be released (from M.ADP; M, myosin) resulting, through the enzyme assay, in NADH breakdown in the solution. Alternatively, extra ATP hydrolysis could be needed during force redevelopment following the release to restore the isometric distribution. The temporal resolution of our measurements does not allow us to discriminate between these possibilities. The estimates of ΔATP_i at 0 and 30 mM P_i, 0·7 and 0·5 ATP molecules per myosin head, respectively, however, are in good agreement with estimates of the fraction of force-producing cross-bridges during an isometric contraction and the influence of $[P_i]$ on that fraction (cf. Potma *et al.* 1995). Therefore, irrespective of the exact origin, we consider it likely that for isovelocity shortenings extra NADH breakdown occurred, not directly associated with the shortening phase, but stoichiometrically coupled to the number of AM.ADP cross-bridges that were forcibly detached prior to the phase of steady shortening. For the isovelocity shortenings this fraction of broken AM.ADP cross-bridges was estimated from the amplitude of force recovery. ΔATP_i obtained from the quick releases, in which for the higher amplitudes all AM.ADP cross-bridges were broken, was multiplied by $(F_e - F_{min})/F_e$ to obtain ΔATP_{corr}. It should be noted that ΔATP found for the 5% release at 0 P_i (55% of ΔATP_i) appears to be low compared with the amount of force which had to be recovered after the release (89% of F_e). However a substantial amount of the force reduction during a 5% quick release was probably elastic and therefore force in that case was not directly proportional to the number of force-producing cross-bridges. Stiffness measurements suggest that to a good approximation this proportionality holds in the case of the semi-steady-state situation during the isovelocity contractions (Julian & Morgan, 1981; Brenner, 1991).

ATP turnover *during* the shortening phase: implications for cross-bridge kinetics

The rate of ATP consumption determines the overall cycling rate and thus probes the rate-limiting cross-bridge transition(s). In fast muscle, during a maximal isometric contraction, detachment from the force-generating AM.ADP state is considered to be the rate-limiting step. During shortening this step is accelerated (cf. Arata *et al.* 1979; Brenner, 1986; Güth *et al.* 1987; Potma *et al.* 1994*a*) and therefore ATP turnover is increased.

To account for the effect of intracellular calcium concentration on the energy turnover during (repetitive) length changes observed by Stienen *et al.* (1993) and by Potma *et al.* (1994*a*) an additional effect of length changes was required, namely that length changes also increase detachment of non- or low-force-producing AM.ADP.P_i cross-bridges, leading to a decrease in ATP turnover. At maximally activating calcium concentration, as was the case in this study, detachment from the force-generating state is the predominant process. Therefore the detachment rate should increase with increasing cross-bridge distortion and thus with increasing shortening velocity.

The effects of $[P_i]$ on isometric ATPase activity may be largely accounted for by mass action of P_i on the reaction from the force-generating AM.ADP state to the non-or-low-force-producing AM.ADP.P_i state (Hibberd *et al.* 1985; Kawai *et al.* 1987; Martyn & Gordon, 1992; Potma *et al.* 1995). It is unlikely that detachment of force-generating cross-bridges is influenced by $[P_i]$ since P_i has no appreciable effect on either unloaded shortening velocity (this study, Cooke & Pate, 1985; Pate & Cooke, 1989*b*) or the rate of tension convergence after photoliberation of caged ATP (Hibberd *et al.* 1985). Also cross-bridge attachment (to the AM.ADP.P_i state) is believed to be independent of $[P_i]$ (cf. Iwamoto, 1995).

The simplest scheme that can account for the observations in this study and our previous findings (Potma *et al.* 1994*a*, *b*, 1995) is the 3-state cross-bridge scheme presented in Potma *et al.* (1995), in which $[P_i]$ acts through mass action on the

AM.ADP to AM.ADP.P_i transition only, and fibre shortening has a moderate effect on detachment of AM.ADP.P_i cross-bridges and a strong effect on detachment of AM.ADP cross-bridges.

Our finding that, below 0·5 L_0 s^{-1}, the ATPase activity during the shortenings was increased by the same factor at 0 and 30 mM P_i, suggests that below 0·5 L_0 s^{-1} the rate of phosphate release is not influenced by shortening. For higher velocities the increase in ATPase activity appears to be higher at high P_i than at low P_i. Simulations of the effects of shortening velocity and [P_i] on the ATPase activity, based on a 3-state cross-bridge scheme, indicate that most of this effect may be explained by mass action. However, at higher velocities an influence of the degree of cross-bridge distortion on the rate of phosphate release (Dantzig *et al.* 1992; Walker *et al.* 1992) may become significant.

Correlations between mechanical performance and energy turnover

The relation between ATP hydrolysis and mechanical performance is reflected by tension cost, the ratio of ATPase activity and force, and efficiency, the ratio of work and energy from ATP hydrolysis. Isometric tension cost at sarcomere length 2·64 μm, Tc_0, was increased between 0 ($n = 16$) and 30 mM P_i ($n = 11$) by a factor of 1·4 ± 0·1. The mean tension cost during the isovelocity shortenings, the (corrected) ATPase activity divided by the mean force during the shortening phase, increased with increasing shortening velocity. At each shortening velocity the ratio of this 'dynamic' tension cost at 0 and 30 mM P_i was the same as for isometric tension cost. Between 0·0625 and 1 L_0 s^{-1} a large increase in dynamic tension cost was found from 2·5 ± 0·2 ($n = 5$) to 15 ± 4 Tc_0 ($n = 7$) at 0 P_i, and from 2·7 ± 0·2 ($n = 5$) to 19 ± 3 Tc_0 ($n = 7$) at 30 mM P_i.

Our observation that during the shortening phase the extra ATP turnover above the isometric rate was proportional to the power output of the preparation, is consistent with heat measurement data during isotonic shortenings (Fenn 1923, 1924; Woledge, Curtin & Homsher, 1985). Consequently, efficiency depends on shortening velocity. The free energy change for ATP splitting (ΔG) amounts to $23RT$ at zero added P_i (where R is the gas constant and T the absolute temperature). Addition of 30 mM P_i causes a reduction in ΔG of $RT \times \ln[2/(30 + 2)] = 2{\cdot}8RT$ ([P_i] ≈ 2 mM in the absence of added phosphate, cf. Stienen *et al.* 1990). Using these values, efficiency can be calculated. Between 0·0625 and 1 L_0 s^{-1}, efficiency increased from 15 to 25% at 0 P_i and from 12 to 23% for 30 mM P_i. Thus efficiency tends to decrease slightly at high [P_i]. In terms of ATP cost, this decrease is somewhat larger. This decline at high [P_i] may contribute to the progressive nature of muscle fatigue at a given (constant) external load.

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