The Effect of Inorganic Phosphate on Force Generation in Single Myofibrils from Rabbit Skeletal Muscle

C. Tesi, F. Colomo, S. Nencini, N. Piroddi, and C. Poggesi
Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, I-50134 Firenze, Italy

ABSTRACT In striated muscle, force generation and phosphate (P_i) release are closely related. Alterations in the [P_i] bathing skinned fibers have been used to probe key transitions of the mechanochemical coupling. Accuracy in this kind of studies is reduced, however, by diffusional barriers. A new perfusion technique is used to study the effect of [P_i] in single or very thin bundles (1–3 μM in diameter; 5°C) of rabbit psoas myofibrils. With this technique, it is possible to rapidly jump [P_i] during contraction and observe the transient and steady-state effects on force of both an increase and a decrease in [P_i]. Steady-state isometric force decreases linearly with an increase in log[P_i] in the range 500 μM to 10 mM (slope -0.4/decade). Between 5 and 200 μM P_i, the slope of the relation is smaller (~ -0.07/decade). The rate constant of force development (k_{TR}) increases with an increase in [P_i] over the same concentration range. After rapid jumps in [P_i], the kinetics of both the force decrease with an increase in [P_i] ($k_{Pi(+)}$) and the force increase with a decrease in [P_i] ($k_{Pi(-)}$) were measured. As observed in skinned fibers with caged P_i, $k_{Pi(+)}$ is about three to four times higher than k_{TR}, strongly dependent on final [P_i], and scarcely modulated by the activation level. Unexpectedly, the kinetics of force increase after jumps from high to low [P_i] is slower: $k_{Pi(-)}$ is indistinguishable from k_{TR} measured at the same [P_i] and has the same calcium sensitivity.

INTRODUCTION

The release of inorganic phosphate (P_i) from the quaternary complex AM.ADP.P_i formed by the contractile proteins (A = actin, M = myosin) and by the products of actomyosin MgATPase reaction (MgADP and P_i) has been associated with force and work production in muscle (for a review see Cooke, 1997). The P_i release step, believed to proceed through a "backdoor" mechanism (for a review, see Geeves and Holmes, 1999), is associated with a large free-energy change (White and Taylor, 1976), is reversible (Sleep and Hutton, 1980; Webb et al., 1986), and marks the transition from myosin states with low affinity for actin (M.ATP, M.ADP.P_i) to strongly binding states (M.ADP, M) (Hibberd and Trentham, 1986; Goldman 1987; Brenner, 1990; Geeves, 1991).

A pivotal role for P_i release in force generation is supported by experiments on demembranated (skinned) fibers. Steady-state studies of skinned fibers from skeletal muscle show that with an increase in [P_i], isometric tension reduces (Brandt et al., 1982; Cooke and Pate, 1985; Kawai et al., 1987; Nosek et al., 1987; Stienen et al., 1990), the rate constants of force development (Millar and Homsher, 1990; Walker *et al.,* 1992) and force release from rigor (Hibberd et al., 1985) increase, and oscillatory work increases (Kawai, 1986). Maximum shortening velocity (Cooke and Pate, 1985; Chase and Kushmerick, 1988) and MgATPase (Webb et al., 1986; Kawai et al., 1987; Cooke et al., 1988) are little affected by P_i. These observations are explained by a reduction of the overall free energy of hydrolysis of MgATP at high [P_i], leading to a net shift of cross-bridges from force-producing states (AM.ADP) to low or no-force-producing states (AM.ADP.P_i), in equilibrium with detached states (Eisenberg *et al.,* 1980; Hibberd and Trentham, 1986). Within this hypothesis, the observed rate of force generation corresponds to the overall rate of the reversible reaction(s) preceding P_i release (i.e., to the sum of the rates of the forward, monomolecular dissociation of AM.ADP.P_i and backward, bimolecular reassociation of AM.ADP and P_i) and is expected to increase with [P_i], as observed. Shortening velocity and MgATPase would not be much affected by [P_i] in conditions where P_i release does not contribute significantly to the rate-limiting step of the overall cross-bridge cycle.

The non-steady-state effect of [P_i] on the kinetics of force generation has been studied by subjecting actively contracting skinned fibers to sudden increases in [P_i] after its photogeneration from caged P_i (as reviewed in Morris and Homsher, 1998). It was shown that the kinetics of the reduction of isometric force caused by the increase in [P_i] 1) depends hyperbolically on final [P_i] (Dantzig et al., 1992; Walker et al., 1992), 2) is faster than the rate constant of force generation at the same [P_i] (Millar and Homsher, 1990; Walker *et al.,* 1992), and 3) is strain dependent (Homsher et al., 1997). From caged P_i experiments and from the effects of [P_i] on force transients evoked by pressure jumps (Fortune et al., 1991, 1994), a two-step mechanism for P_i release has also been suggested, with a force-generating isomerization of AM.ADP.P_i preceding the release of P_i.

Studies of the effects of [P_i] on the force generation mechanism in skinned fibers are complicated by diffusional barriers that, especially during active contraction when the

Received for publication 16 November 1999 and in final form 11 February 2000.

Address reprint requests to Dr. Chiara Tesi, Dipartimento di Scienze Fisiologiche, Università degli studi di Firenze, viale G. B. Morgagni 63, I-50134 Firenze, Italy. Tel.: 39-55-4237341; Fax: 39-55-4379506; E-mail: chiara.tesi@unifi.it.

0006-3495/00/06/3081/12 $2.00

rate of MgATP hydrolysis is high, reduce control over substrate and product concentrations inside the myofilament lattice. For these reasons, a modeling approach is required to interpret mechanical data from skinned fibers in low [P_i], which is essential for predictions of actomyosin states in the powerstroke (Cooke and Pate, 1985; Pate et al., 1998). For these reasons, caged compounds are needed to obtain fast perturbations of ligand concentration. With the release of P_i from caged P_i, mechanical transients can be observed only as decreases in force.

Recently it has been shown that the limitations of diffusional delays in skinned fibers can be overcome by the use of single myofibrils, a preparation small enough for biochemical studies and suitable for mechanical measurements (Bartoo et al., 1993; Friedman and Goldman, 1996; Colomo et al., 1997; Barman et al., 1998). In particular, myofibrils (1–3 μm wide) are in rapid diffusional equilibrium with the bathing medium, so that tight control of the solution composition in the myofilament lattice is possible with rapid changes in the bathing solution (Tesi et al., 1999). The development of systems for rapid solution change around myofibrils made it possible to resolve the kinetics of force rise and relaxation in an activation cycle (Colomo et al., 1998; Nencini et al., 1999) and the transient force perturbations initiated by rapid changes in substrate and product concentrations (Tesi et al., 1999).

Here single myofibrils or thin bundles of myofibrils from rabbit skeletal muscle, activated in different conditions by rapid alternation of the bathing media, have been used to study the effects of [P_i] on 1) maximum isometric force, 2) the rate of force redevelopment after a release-restretch protocol (k_{TR}; Brenner, 1988), and 3) the kinetics of force transients initiated by sudden modifications of [P_i]. In particular, the force/[P_i] and k_{TR}/[P_i] relations were determined down to 5 μM, without the use of diffusional models, and the kinetics of the force increase in response to a sudden decrease in [P_i] has been described for the first time. Preliminary results from these studies have been reported (Piroddi et al., 1999).

MATERIALS AND METHODS

Myofibrils

Single myofibrils or thin bundles of myofibrils were prepared from rabbit fast skeletal muscle by homogenization of glycerinated rabbit psoas muscle, as previously described (Tesi et al., 1999). After homogenization, myofibrils were washed twice in rigor solution, recovered by low-speed centrifugation (2000 $\times$ g for 10 min 0–4°C), kept in ice, and used within 12 h. For the experiments, a small volume of myofibril suspension was transferred to a temperature-controlled trough (5°C) mounted on an inverted microscope (Nikon, Diaphot, Japan) and filled with relaxing solution (pCa 8). In some experiments, myofibrils from rabbit soleus muscle isolated following the same procedure were used. The initial length (l_o) of the preparation was set 5–10% above the slack myofibril length. Mean initial sarcomere lengths (s.l.), measured on video images (4000×; phase-contrast optics) were 2.60 $\pm$ 0.02 μm (n = 79) and 2.50 $\pm$ 0.02 μm (n = 9) in rabbit psoas and soleus muscle myofibrils, respectively.

Experimental apparatus for force recording and rapid solution change

The method we used to record isometric force from single striated muscle myofibrils and the system developed for rapid solution change have been described (Colomo et al., 1997, 1998). Briefly, myofibrils selected for experiments were mounted horizontally between two glass microtools. One tool served as a calibrated force probe. The other tool was connected to the lever arm of a length control motor. The preparations strongly adhered to the glass tools, which were micromanipulated to maximize the attachment area. The force probe used in the present experiments had a compliance of 1–3 nm nN^{-1} and a frequency response of 2–5 kHz in the experimental solutions. Isometric force was measured by photoelectronically recording the elastic deflection of the force probe; myofibril shortening due to force-probe compliance was kept below 3% of the initial length.

Myofibrils were maximally activated and relaxed by rapid translation between two continuous streams of relaxing (pCa 8.0) and activating (pCa 4.5) solutions of variable composition, jetted by gravity from a double-barreled glass pipette placed at a right angle with the preparation (1 mm distance). The gravity-driven flow rates of the two solutions were equal and were ~60 μl/min, which resulted in estimated speeds of the solution flow past the myofibril of ~2 cm/s. The time course of the solution change resulting from the displacement of the perfusion pipette was estimated by placing two microelectrodes in the solution stream as close to the position of the ends of mounted myofibrils as possible, passing current between them, and measuring the change in current concomitant with switching between two solutions of different KCl concentrations. Solution change took place with a time constant of 2–4 ms and was complete in ~10 ms. Solution change after the start of the pipette movement was delayed by a time (usually 50 ms) that depended on the velocity of the solution flow and on the distance between the mouth of the perfusion pipette and the myofibril.

During experiments myofibrils were subjected to several activation-relaxation cycles by switching the position of the perfusion pipette as described above. To compare maximum isometric force in two different experimental conditions, myofibrils were sequentially activated, using a translating holder carrying two perfusing systems (four streams). To further increase the number of parallel fluxes of the perfusing system, we replaced double-barreled glass pipettes with fused silica chromatography columns (guard column IP-deact., 100–200 μm diameter; Restek) glued side by side, and each channel was directly connected to a solution reservoir.

Contractions were usually well reproduced over four or five activation cycles before there was a significant decline in the mechanical performance of myofibrils (i.e., more than a 10% decrease in isometric force and in speed of force development).

Experiments with rabbit psoas myofibrils were performed at 5°C and with rabbit soleus myofibrils at 15°C and 20°C.

Release-restretch protocols applied to myofibrils at the contraction plateau were used to measure the time course of force redevelopment (Brenner, 1988). Briefly, a sudden decrease in length (10–20% l_o) was imposed on myofibrils during a steady contraction, bringing the initial length of the preparation below the slack value. After a period of unloaded shortening, which reduced the number of cross-bridges to a minimal value and before unloaded myofibrils could take up the slack (35–40 ms), myofibrils were rapidly stretched back to their original length to mechanically dissociate the residual acto-myosin bonds. After restretch, force redeveloped to the isometric plateau with a time course that reflects the kinetics of strong cross-bridge attachment and force generation (Brenner, 1988).

Solutions: composition and reduction of contaminant P_i

All solutions, calculated as previously described (Brandt et al., 1998), contained 5 mM MgATP, 1 mM free magnesium (pH 7.0), and a final ionic

strength of 200 mM. Solutions of different pCa levels were calculated by solving the multiple equilibria for metals and ligands, using the following apparent association constant (log values at pH 7.00): CaEGTA 6.3, MgEGTA 1.6, CaATP 3.7, MgATP 4.1. Although continuous solution flow minimizes alterations in the concentration of MgATP and its hydrolysis products in the myofibrillar space, the present measurements have been made in the presence of a MgATP regenerating system: creatine phosphate (CP = 10 mM) and creatine kinase (CPK = 200 units ml^{-1}). As previously observed (Tesi et al., 1999), the presence of this rephosphorylating system reduces the rundown of preparations.

Actual $[P_i]$ in experimental solutions was spectrophotometrically estimated (Baginski et al., 1967) on samples collected before the addition of CPK, the presence of which interfered with the colorimetric assay. Standard solutions contain some contaminating P_i (mainly coming from spontaneous breakdown of MgATP and CP), which is estimated to be 170 μM ± 30 (n = 17). When used, these solutions are referred to as "nominal P_i " solutions.

Contaminant P_i in experimental solutions was reduced by employing the enzyme purine nucleoside phosphorylase (PNP) with the substrate 7-methyl guanosine (Brune et al., 1994), immobilized by coupling to cyanogen bromide-activated Sepharose 4B beads. When the solutions, added with the substrate, are passed through columns packed with the enzyme-coupled beads, P_i contamination is reduced to less than 5 μM. When used, these solutions are referred to as "P_i-free" solutions.

Contractions recorded from myofibrils perfused with P_i-free solutions were generally used as a reference. Test contractions at any $[P_i]$ are obtained using solutions first scavenged for P_i and subsequently added with known amounts of P_i ("test solutions").

In some control experiments (see Discussion) solutions containing 3 mM MgADP were used. The apparent association constants (log values) used for calculations were 3.1 and 2.7 for MgADP and CaADP, respectively. Creatine phosphate and creatine kinase were omitted from MgADP solutions and from control solutions (no MgADP added) used for those experiments. Both solution types were scavenged for contaminating P_i (see above).

Nucleoside phosphorylase ("bacterial"), 7-methylguanosine, ATP, ADP, and CP/CPK were purchased from Sigma Chemical Co.

Results are given as means ± SE.

RESULTS

Effects of $[P_i]$ on force and rate of force generation in maximally activated rabbit psoas myofibrils

Single myofibrils or thin bundles of two to four myofibrils from rabbit psoas muscle were maximally activated and relaxed at 5°C in the presence of variable $[P_i]$ by rapid solution change. Throughout the experiments, the concentrations of P_i and other solutes inside the myofibril lattice were assumed to be equal to that of the perfusing solutions. In each experiment $[P_i]$ was colorimetrically assayed (see Materials and Methods). The small diameter of the preparations (1–3 μm) and the continuous renewal of solutions prevented the establishment of significant diffusional gradients and made corrections of $[P_i]$ based on diffusional models (Pate and Cooke, 1989a) unnecessary with myofibrils (less than 1 μM).

As widely observed in whole fibers, the addition of P_i to nominal solutions decreased isometric steady-state force and increased the rate of force development of rabbit psoas myofibrils (Fig. 1). Information about the rate of force generation was obtained from both the time course of force rise after rapid activation (Fig. 1 *B*) and from the time course of force redevelopment after a release-restretch protocol (Fig. 1 *C*). The half-time (t_{50}) of force development after activation was very close to that of force redevelopment, though systematically 5–10% longer. Where not otherwise stated, the apparent rate of force generation (k_{TR}) was estimated from the half-time of force redevelopment phase, assumed to be a monoexponential process. The mechanical properties of isolated myofibrils were highly sensitive to $[P_i]$: 5 mM P_i reduced isometric force to less than 50% and speeded up force rise by about three times compared to values obtained in solutions containing only contaminant P_i (~170 μM).

To reduce P_i contamination and investigate the effects of low concentrations of ligand, experimental solutions were treated with immobilized PNP in the presence of 7-methylguanosine. Such "P_i-free" solutions ($[P_i] < 5$ μM) were then taken as the reference condition and used to prepare test solutions with the addition of known amounts of P_i. Mean maximum isometric force of myofibrils in P_i-free solution was 862 ± 44 nN, corresponding to a mean tension of 265 ± 14 mN/mm^2 (n = 36), while the half-time of force generation was 363 ± 9 ms (k_{TR} 1.9 ± 0.1 s^{-1}; n = 36). The relation between $[P_i]$ and relative steady-state force is shown in Figs. 2 *A* and 3 *A*. Values are paired observations from 5–36 experiments normalized to control "P_i-free" conditions. To facilitate comparison with previous work (Pate and Cooke, 1989a,b; Millar and Homsher, 1990; Dantzig et al., 1992; Pate et al., 1998), P_i concentration has been plotted in Fig. 2 on a logarithmic scale, as a linear relation is expected based on energetic considerations. As previously observed in skinned fibers, above 500 μM P_i force decreased linearly with $\log[P_i]$ (slope: −0.40 relative force unit per decade increase of $[P_i]$). Below 200 μM P_i the sensitivity of force to $\log[P_i]$ was much smaller (slope: −0.07 relative force unit per decade increase of $[P_i]$). The force-$[P_i]$ relation of rabbit psoas myofibrils can be equally well fit by a hyperbola on a linear scale (Fig. 3 *A*), giving a Pi_{50} of 4.2 ± 0.5 mM.

Above 200 μM $[P_i]$, half-time of force development after activation increases linearly with $\log[P_i]$ (Fig. 2 *B*). As shown in Fig. 3 *B*, the hyperbolic fit of the plot of absolute k_{TR} versus $[P_i]$ gave a P_{i50} of 7.8 ± 2.5 mM.

Effects of perturbations of $[P_i]$ on force in maximally activated rabbit psoas myofibrils

As expected from the effects of $[P_i]$ on steady-state isometric force development in striated muscle and from previous observations of force transients after caged P_i photolysis in skinned fibers, myofibrils from rabbit psoas muscle responded with sudden changes in maximum isometric force to the imposition of rapid changes in $[P_i]$ obtained by fast

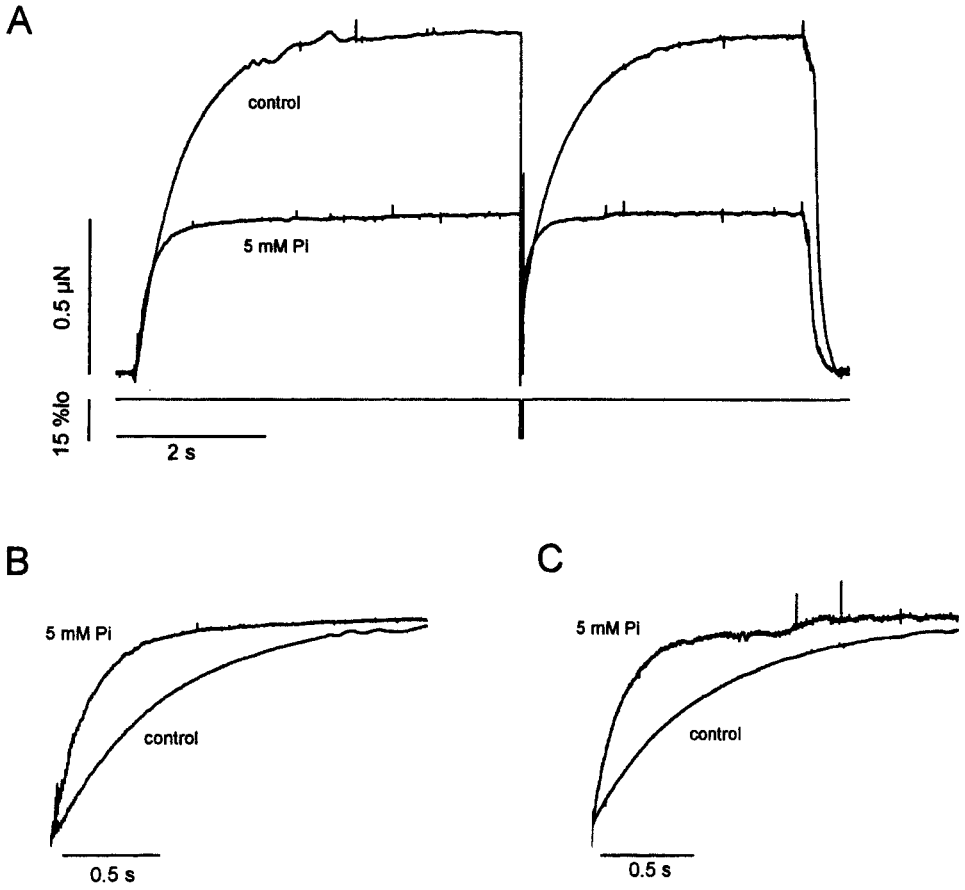


FIGURE 1 Effects of 5 mM P_i addition on a single rabbit psoas myofibril during a contraction-relaxation cycle (5°C; l_o 52 μm; s.l. 2.5 μm). (*A*) A concentration of 5 mM P_i dropped the isometric force to 0.46 of force in nominal solution (contaminant $[P_i]$ 170 μM). The effects of $[P_i]$ on the time course of force development (*B*) and on the time course of force redevelopment after a release-restretch protocol (*C*) are better resolved after force normalization. In control conditions the t_{50} of force development and redevelopment is 450 ms and 430 ms, respectively. In 5 mM $[P_i]$ the t_{50} of force development and redevelopment is 160 and 140 ms, respectively.

alternation of the perfusing flux. In the simplest P_i jump protocol we followed (Fig. 4 *A*), myofibrils were activated in low $[P_i]$ solutions, and once a steady plateau of isometric force was attained, the perfusing flux was rapidly switched to a high $[P_i]$ solution and back. Force transients resulting from both an increase and a decrease in $[P_i]$ were then recorded, and their rates (named $k_{Pi(+)}$ and $k_{Pi(-)}$, respectively) were estimated from the observed half-time of force changes.

As shown in Fig. 4 *A*, when $[P_i]$ was increased from 0.1 mM to 5 mM, force dropped to a value close to what expected from the steady-state isometric force/$[P_i]$ curve, and the rate constant of P_i transient $k_{Pi(+)}$ was 28 s^{-1}, i.e., about four times faster than the average k_{TR} measured at the same ligand concentration (6.9 ± 1 s^{-1}, $n = 7$; Fig. 3 *B*, point at 5 mM P_i). When we consider the lower temperature of our experiments (see the Discussion), the $k_{Pi(+)}$ value observed in rabbit psoas myofibrils is close to that expected from caged P_i experiments at the same final $[P_i]$. Interestingly, after the reversal of the P_i jump from 5 mM to 0.1 mM $[P_i]$, the rate of the transient force increase observed here ($k_{Pi(-)} = 1.7$ s^{-1}) was much slower and indistinguishable from k_{TR} at the corresponding final $[P_i]$ (2.2 ± 0.2 s^{-1}, $n = 5$; Fig. 3 *B*, point at 0.1 mM $[P_i]$). As P_i transient kinetics depends on the final ligand concentration (Dantzig et al., 1992; Walker et al., 1992), the apparent rate of the force transition measured at 0.1 mM $[P_i]$ is expected to be slower than at 5 mM P_i, but in any case still significantly higher than k_{TR}. This unexpected behavior was not dependent on the experimental protocol, as a lower value of $k_{Pi(-)}$ compared to $k_{Pi(+)}$ was also observed when the sequence of force jumps was reversed (Fig. 4 *B*).

To investigate the asymmetrical kinetics of force transitions obtained after an increase or a decrease in $[P_i]$, we performed a series of P_i jumps to the same final level (5 mM) by starting in turn from higher (10 mM) or lower (0.1 mM) $[P_i]$. In each test, we also measured k_{TR} at the reference final $[P_i]$, by performing a release-restretch protocol as soon as a new steady force was attained. The results of these experiments (Table 1)

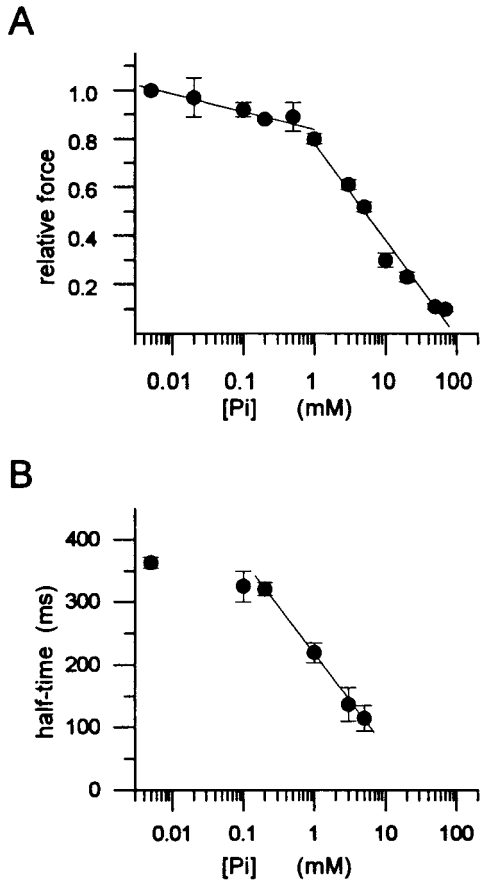


FIGURE 2 (*A*) Tension relative to that obtained in "P_i-free" conditions ($[P_i]$ below 5 μM). (*B*) Absolute half-time of force generation (t_{50}) as a function of log$[P_i]$. Straight lines are linear regressions. In *A* the slope of linear regressions below 200 μM and above 500 μM P_i is -0.07/decade increase $[P_i]$ and -0.40/decade increase $[P_i]$, respectively. In *B* the slope of linear regression above 200 μM P_i is -149 ms/decade increase $[P_i]$, corresponding to -0.41 relative unit/decade increase [Pi] (t_{50} in P_i-free conditions: 363 $\pm$ 9 ms, $n = 36$). Each point represents mean values obtained from 5–36 myofibrils.

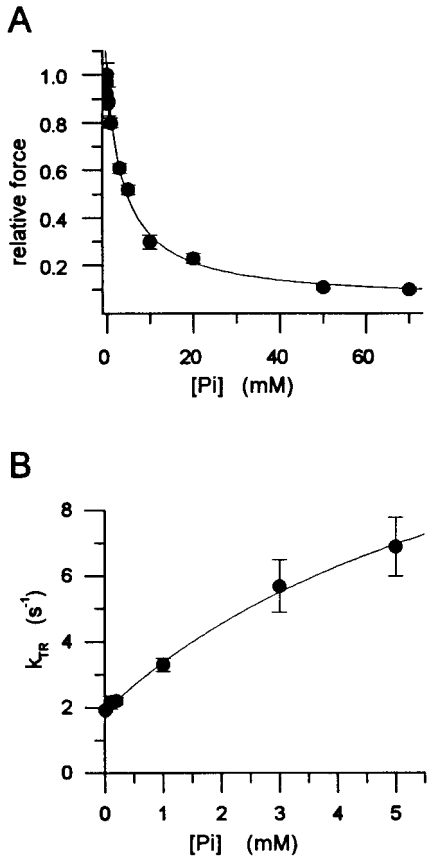


FIGURE 3 Hyperbolic fits of plots of relative force (*A*) and absolute k_{TR} (*B*) data against $[P_i]$. In *A* the Pi_{50} for force depression is 4.2 $\pm$ 0.5 mM. In *B* the Pi_{50} for k_{TR} acceleration is 7.8 $\pm$ 2.5 mM, the maximum k_{TR} is 13 $\pm$ 2.7 s^{-1}, and the intercept is 1.9 $\pm$ 0.1 s^{-1}.

confirm that the rates of force transitions observed after P_i jumps depended not only on the final $[P_i]$ but also on the direction of $[P_i]$ change, $k_{Pi(-)}$ being three or four times slower than $k_{Pi(+)}$ and not significantly different from k_{TR}.

Preliminary jump experiments showed that both $k_{Pi(+)}$ and $k_{Pi(-)}$ did not depend on the initial $[P_i]$. For instance, $k_{Pi(+)}$, measured from 1 to 5 mM P_i, was 16.1 $\pm$ 1.9 s^{-1} ($n = 11$), i.e., very close to the value measured from 0.1 to 5 mM (see Table 1). Similarly, $k_{Pi(-)}$ values, measured from 1 or 5 to 0.1 mM P_i, were not different (1.8 $\pm$ 0.1 s^{-1} and 1.7 $\pm$ 0.1 s^{-1}, respectively; $n = 5$).

Calcium sensitivity of the kinetics of force changes induced by P_i jumps

In our experiments the rate of force transients induced by a sudden decrease in $[P_i]$ ($k_{Pi(-)}$) was always found to be very close to k_{TR} at the same final $[P_i]$. We further tested this observation by studying the effect of calcium concentration on the kinetics of P_i-transients in myofibrils. It is known that calcium concentration affects the kinetics of force generation in striated muscle (Brenner, 1988), and the Ca^{2+} dependency of k_{TR} has recently been confirmed in single myofibrils (Colomo et al., 1998). In contrast, the kinetics of P_i-transients induced by photolysis of caged P_i is relatively insensitive to $[Ca^{2+}]$ (Millar and Homsher, 1990; Walker et al., 1992). If $k_{Pi(-)}$ is limited by the same transition as k_{TR}, we expect $k_{Pi(-)}$ to be strongly Ca-sensitive and the difference between $k_{Pi(-)}$ and $k_{Pi(+)}$ to increase at submaximum activating calcium.

To test this, we submitted single psoas muscle myofibrils (Fig. 5) to the same P_i jumps, from 0.1 mM to 5 mM P_i and back, at maximum and submaximum calcium (pCa 4.5 and pCa 5.75). At pCa 5.75 in reference conditions (0.1 mM P_i), both isometric force and k_{TR} were strongly depressed, being 0.64 $\pm$ 0.06 and 0.46 $\pm$ 0.05 ($n = 8$) of their maximum activated values, respectively.

From the experimental traces in Fig. 5, it can be seen that lowering $[Ca^{2+}]$ strongly affected $k_{Pi(-)}$, which decreased

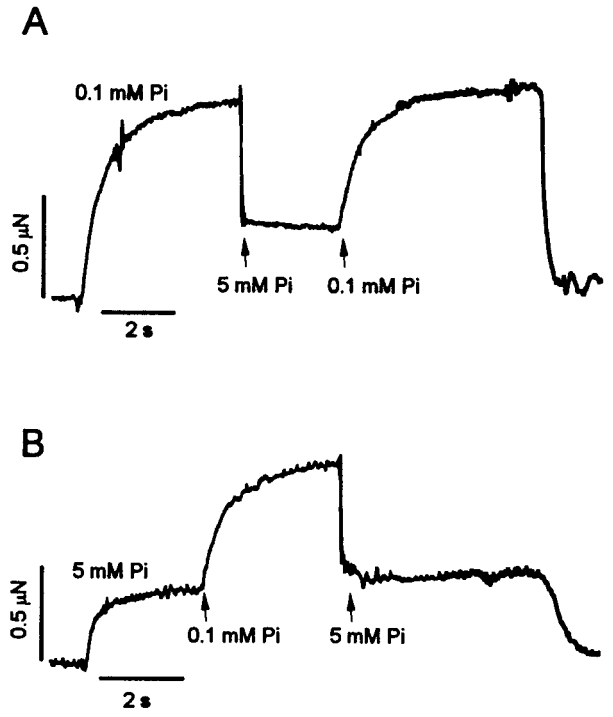


FIGURE 4 (*A*) Force response of a rabbit psoas myofibril activated in low-P_i solution and subjected to a $[P_i]$ jump to 5 mM (*first arrow*) that drops force to a constant level that is maintained until $[P_i]$ is jumped back to 0.1 mM (*second arrow*). P_o 5 mM/P_o 0.1 mM = 0.36. For the force rise following activation: t_{50} = 450 ms; for the P_i jumps: 0.1 mM to 5 mM, $k_{Pi(+)}$ = 27.7 s^{-1} (t_{50} = 25 ms); 5 mM to 0.1mM $[P_i]$, $k_{Pi(-)}$ = 1.7 s^{-1} (t_{50} = 400 ms). (*B*) Similar P_i-transients obtained when the same myofibril was first activated in the presence of 5 mM P_i and then subjected to a sudden decrease in $[P_i]$ to 0.1 mM, leading to a force increase and then a decrease. P_o 5 mM/P_o 0.1 mM = 0.35. For the force rise following activation: t_{50} = 160 ms; for the P_i jumps: 5 mM to 0.1 mM $[P_i]$, $k_{Pi(-)}$ = 1.5 s^{-1} (t_{50} = 450 ms); 0.1 mM to 5 mM, $k_{Pi(+)}$ = 25.7 s^{-1} (t_{50} = 27 ms). Rabbit psoas myofibril, l_o = 92 μm; *s.l.* = 2.5 μm.

by almost 50%. Mean $k_{Pi(-)}$ at pCa 4.5 and at pCa 5.75 was 1.71 ± 0.11 s^{-1} and 1.04 ± 0.18 s^{-1}, respectively (same experiments as above, n = 8). The effect of calcium on $k_{Pi(-)}$ and k_{TR} was quantitatively the same (see above), and the kinetics of force increase after a release-restretch protocol and after a sudden decrease in $[P_i]$ were similar at maximum and submaximum activation (Fig. 5, *right panels*). The ratio $k_{TR}/k_{Pi(-)}$ did not significantly deviate from 1 at both pCa tested, being 1.15 ± 0.11 (p = 0.22) at pCa 4.5 and 0.95 ± 0.06 (p = 0.43) at pCa 5.75.

In agreement with Millar and Homsher's observations in skinned fibers (Millar and Homsher, 1990), $k_{Pi(+)}$ in myofibrils showed no detectable dependence on activation level, being 18.2 ± 2.0 s^{-1} at pCa 4.5 and 20 ± 2 s^{-1} at pCa 5.75.

Experiments on rabbit soleus muscle myofibrils

To increase the time resolution of P_i jumps, experiments were performed using rabbit soleus muscle myofibrils (20°C and 15°C), which have a much slower rate of force generation than rabbit psoas (Fig. 6). For example, at 15°C (free P_i solutions) the mean half-time for force development after rapid activation of soleus myofibrils was 353 ± 28 ms (k_{TR} = 2.1 ± 0.1 s^{-1}, n = 11), i.e., three to four times longer than that for psoas myofibrils at the same temperature: 101 ± 10 ms (k_{TR} 7.2 ± 0.6 s^{-1}, n = 6). It has been shown that the slower rate of force generation in soleus muscle corresponds to slower P_i release (Millar and Homsher, 1992; Wahr et al., 1997).

In P_i jump experiments with soleus myofibrils, a four-channel perfusion pipette was used that enabled us to measure both $k_{Pi(+)}$ and $k_{Pi(-)}$ in a single contraction at the same final $[P_i]$ (Fig. 7). Briefly, myofibrils maximally activated with a P_i-free pCa 4.5 solution were subjected to three sequential P_i jumps, first to 2 mM and then to 20 mM $[P_i]$, followed by a last P_i jump back to 2 mM (Fig. 7 *A*). At each $[P_i]$, k_{TR} was estimated by a release-restretch protocol. The results show that the kinetics of the P_i transient for soleus myofibrils at 20°C clearly depends on the path followed to reach the final $[P_i]$, being faster when $[P_i]$ was suddenly increased. Also, as in psoas myofibrils, $k_{Pi(+)}$ was three to four times higher than $k_{Pi(-)}$, which again was about the same as k_{TR} (see Table 1).

With soleus myofibrils, $k_{Pi(+)}$ observed at 2 mM $[P_i]$ (*fourth column* in Table 1) is two to three times higher than that previously determined in caged P_i experiments on skinned soleus fibers (~5 and ~1 s^{-1} at 20°C and 15°C, respectively; Millar and Homsher, 1992) or by sinusoidal analysis (3.5 at 20°C; Wang and Kawai, 1997). Interestingly, the rate of force development we measured in soleus myofibrils is also two to three times higher than previous estimates for skinned fibers (2.32 ± 0.24 s^{-1} at 1 mM P_i and 20°C; Millar and Homsher, 1992).

In agreement with previous observations (Wahr et al., 1997), force developed by slow muscle was depressed by $[P_i]$ to a lesser extent than in fast muscle: at 20 mM P_i relative force in soleus myofibrils was 0.65 ± 0.03 at 20°C (n = 12) and 0.56 ± 0.02 at 15°C (n = 6), while in rabbit psoas myofibrils at 5°C it was ~0.25. No clear acceleration of k_{TR} with $[P_i]$ was observed in the range from P_i-free to 20 mM $[P_i]$. Interestingly, at 15°C, the P_i transients became less P_i-sensitive and more symmetrical than at 20°C and, similar to observations in previous caged P_i experiments on skinned soleus fibers, the difference between $k_{Pi(+)}$ and k_{TR} was much reduced. This confirms the enormous temperature dependence of the kinetics of P_i transients in soleus muscle (Millar and Homsher, 1992).

DISCUSSION

The use of skeletal muscle myofibrils activated by rapid solution exchange offers important advantages over larger preparations in the studies of steady-state and non-steady-state effects of $[P_i]$ on force generation in striated muscle.

TABLE 1 Kinetics of P_i transients and of force redevelopment in myofibrils of rabbit psoas and soleus muscle

	Psoas			Soleus		
T (°C)	$k_{Pi(+)}$ (s^{-1}) 0.1 mM → 5 mM	$k_{Pi(-)}$ (s^{-1}) 10 mM → 5 mM	k_{TR} (s^{-1}) 5 mM	$k_{Pi(+)}$ (s^{-1}) 5 μM → 2 mM	$k_{Pi(-)}$ (s^{-1}) 20 mM → 2 mM	k_{TR} (s^{-1}) 2 mM
5	18.2 ± 2.0	5.2 ± 0.7	5.4 ± 0.6			
15				3.2 ± 0.6	1.8 ± 0.2	1.8 ± 0.2
20				11.3 ± 0.7	3.2 ± 0.2	4.1 ± 0.4

$k_{Pi(+)}$, rate constants of force decline initiated by an increase in $[P_i]$; $k_{Pi(-)}$, rate constants of force increase initiated by a decrease in $[P_i]$; k_{TR}, rate constants of force generation measured from release-restretch protocols performed after the jumps. Values are mean ± SE obtained in rabbit psoas myofibrils at 5 mM final $[P_i]$ ($n = 13$) or in rabbit soleus myofibrils at 2 mM final $[P_i]$ (15°C: $n = 6$; 20°C: $n = 12$).

Steady-state isometric force-$[P_i]$ and k_{TR}-$[P_i]$ relations were determined for a wide range of $[P_i]$, also in the submillimolar range, without the need for diffusional models to derive the actual $[P_i]$ inside the myofibrillar lattice. The results confirmed and extended studies performed in skinned fibers, where resolution was limited by diffusional artifacts. The effects on isometric force of sudden perturbations of $[P_i]$ could be observed for the first time, also on decreasing $[P_i]$ jumps. The comparison of force responses obtained after P_i jumps to the same final $[P_i]$, but starting from higher or lower initial $[P_i]$, showed a large asymmetry in their kinetics, which was unexpected from predictions based on chemomechanical coupling models of muscle contraction.

Steady-state effects of $[P_i]$

The present study confirms many previous observations reporting a nonlinear relationship between isometric force and $[P_i]$ in skinned fibers (reviewed in Pate et al., 1998). This relation can be equally well fit by exponential or hyperbolic functions (Stienen et al., 1990), but the first is

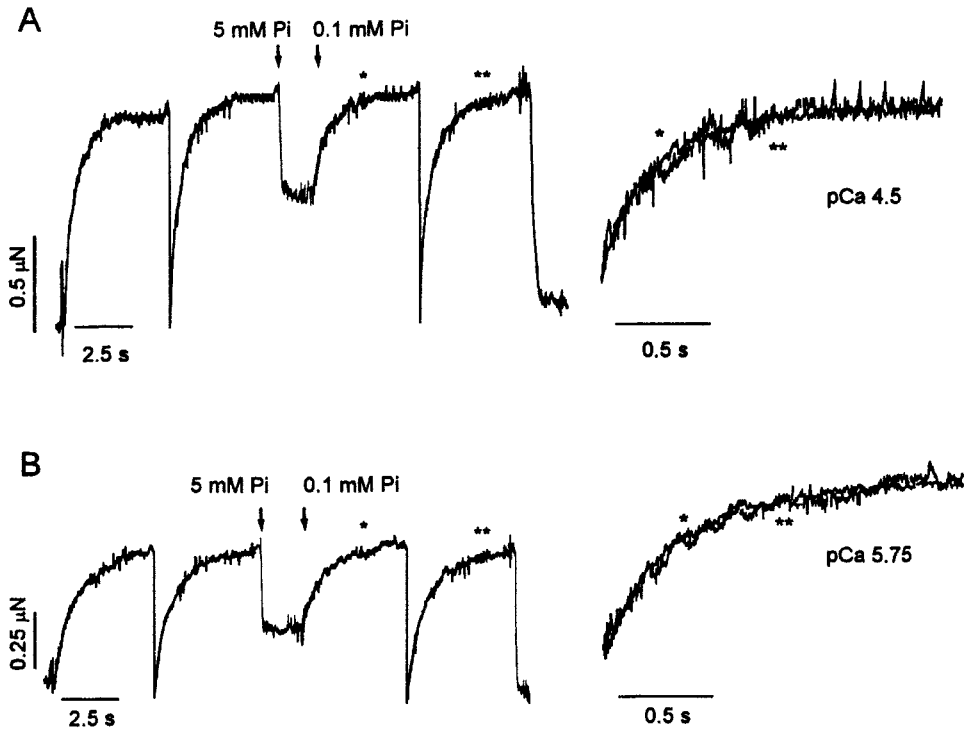


FIGURE 5 Force changes initiated by P_i jumps from 0.1 mM to 5 mM (*first arrow*) and back (*second arrow*) in pCa 4.5 (*A*) and 5.75 (*B*). For both activation levels, the time courses of the force increase that followed a sudden decrease in $[P_i]$ to 0.1 mM (*) is very close to that of force redevelopment measured at the same final $[P_i]$ (**), as shown by the close correspondence of superimposed traces (*A* and *B*, *right panels*). Full activation (*A*): $k_{Pi(+)} = 11.9\ s^{-1}$ for 5 mM P_i jump; $k_{Pi(-)} = 1.7\ s^{-1}$ for 0.1 mM P_i jump; $k_{TR} = 1.8\ s^{-1}$ for force redevelopment in 0.1 mM P_i. pCa 5.75 (*B*): $k_{Pi(+)} = 17.3\ s^{-1}$ for 5 mM P_i jump; $k_{Pi(-)} = 1.1\ s^{-1}$ for 0.1 mM P_i jump; $k_{TR} = 1.3\ s^{-1}$ for force redevelopment in 0.1 mM P_i. Rabbit psoas myofibril, $l_o = 54$ μm; *s.l.* = 2.5 μm.

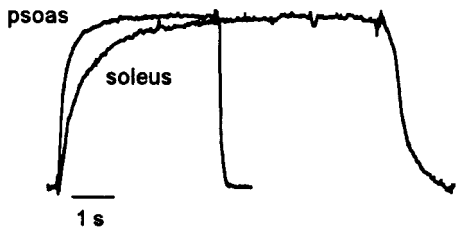


FIGURE 6 Activation-relaxation cycles of soleus and psoas myofibrils at 15°C. For psoas: P_o = 1130 nN and t_{50} of force development is 93 ms (two or three myofibril bundles). For soleus: P_o = 630 nN and t_{50} of force development is 360 ms (one or two myofibril bundles).

usually chosen based on thermodynamic considerations and the Hill formalism (Eisenberg et al., 1980; Hibberd and Trentham, 1986; Pate and Cooke, 1989b).

The slope of the force/log[P_i] relation for rabbit psoas myofibrils above 200 μM is −0.4 relative force units per [P_i] decade increase, a value that is situated in the upper range of previous observations (Cooke and Pate, 1985; Pate and Cooke, 1989a; Millar and Homsher, 1990; Dantzig et al., 1992; Martyn and Gordon, 1992; Wilson et al., 1995; Wahr et al., 1997; Pate et al., 1998, Regnier et al., 1998). The high slope of the force/log [P_i] relation obtained here, using a single myofibril, is expected from the lower temperature of our experiments (5°C) compared to the previous range (10–20°C; see figure 1 in Dantzig et al., 1992) and because the internal P_i of myofibrils is virtually undamped (diffusional barriers are minimal).

To reduce [P_i] in the lattice space of skinned fibers, phosphate scavenging enzymatic systems have been used (sucrose phosphorylase/sucrose; Pate and Cooke, 1989a; Millar and Homsher 1990; Martyn and Gordon, 1992). In a recent work Cooke, Pate, and collaborators succeeded in reducing [P_i] to less than 5 μM (with a nucleoside phosphorylase/7-methylguanosine system; Pate et al., 1998). A considerable agreement exists between our low [P_i] data and those of Pate et al., the only difference is that we observed a decrease of five times in the slope of the force/log[P_i] relation of myofibrils below 200 μM (−0.07 relative force units per decade increase [P_i]), while Cooke, Pate, and collaborators report a constant slope down to 100 μM P_i and then a force plateau at lower [P_i]. Hyperbolic fitting of relative force versus [P_i] in myofibrils gave a Pi_{50} for force depression of 4.2 ± 0.5 mM. This value, again, is in the range of those previously observed (3–12 mM; Cooke and Pate, 1985; Wilson et al., 1995) and not far from the Pi_{50} for P_i incorporation into MgATP measured in fibers by ^{18}O exchange (3 mM; Bowater and Sleep, 1988).

The maximum depression of isometric force in high [P_i] that we observed at 5°C in rabbit psoas myofibrils is somewhat greater than that obtained at 10–20°C in skinned psoas fibers. For example, at 20 mM [P_i], we report a relative isometric force of ~0.25 (see Figs. 2 and 3 *A*), while the range of previous observations at the same ligand concentration extended from ~0.6 (Martyn and Gordon, 1992, 10°C; Wang and Kawai, 1997, 20°C;) to ~0.4 (Millar and Homsher, 1990, 10°C; Wahr et al., 15°C), with most observations clustered around 0.5. This is expected from the absence of diffusional barriers in single myofibrils and from the relation between the diameter of the preparation and the depressant effect of phosphate on isometric force (Stienen et al., 1990; see Fig. 4). Moreover, our maximum control forces were probably relatively higher than those obtained in previous studies because of the near absence of P_i contamination in our reference conditions.

In agreement with previous observations on skinned fibers (Millar and Homsher, 1990; Metzger and Moss, 1990; Regnier et al., 1995; Regnier and Homsher, 1998; Wahr et al., 1997), [P_i] strongly modulated the rate constant of force generation in myofibrils (measured from the kinetics of force redevelopment after a release-restretch protocol). k_{TR} increased almost three times over the range of [P_i] tested (Fig. 3 *B*). The hyperbolic k_{TR}/[P_i] relation yields a Pi_{50} of 7.8 ± 2.5 mM. This is very close to the corresponding value for the relative force/[P_i] relation. Modulation by [P_i] of both isometric force and k_{TR} (but with opposite signs) is expected from models of the cross-bridge cycle that link P_i release with the driving stroke and then steady-state force to the fraction of cross-bridges in strongly bound (AM.ADP or AM) states. According to these models (Brenner, 1988) k_{TR} is thought to report the apparent rate of transition from detached or weakly bound states to force-generating states and thus includes the rates for strong cross-bridge attachment, P_i release, and force generation. An increase in [P_i], accelerating the reversal of the driving stroke, would at the same time increase k_{TR}, which is represented by the sum of the forward and reverse rate constants of the elementary steps, and shift the distribution of cross-bridges toward the no-force states (for a review see Goldman, 1987). Interestingly, the difference that we observed between the values of Pi_{50} of the force/[P_i] and that of the k_{TR}/[P_i] relation (Pi_{50} is higher for k_{TR} than for force) is predicted by computer simulations with the Millar and Homsher model and the rate constants and procedure described by Regnier et al. (1995).

The kinetics of P_i jumps

The kinetics of the force transients observed in single rabbit myofibrils with P_i jumps can only be compared with observations on skinned fibers where decreases in force are elicited by photoliberation of caged phosphate. Before the present report there were no measurements of the kinetics of force increase after a sudden decrease in [P_i].

The mean value of $k_{Pi(+)}$ for rabbit psoas myofibrils at 5 mM final [P_i] (5°C) is 18.4 ± 2.1 s^{-1} (n = 13; Table 1); in skinned fibers from the same muscle (10°C) it ranges from 30 s^{-1} (Regnier and Homsher, 1998) to 60 s^{-1} (Dantzig et al., 1992). Assuming a Q_{10} of ~3.5 (Dantzig et al., 1992; Walker

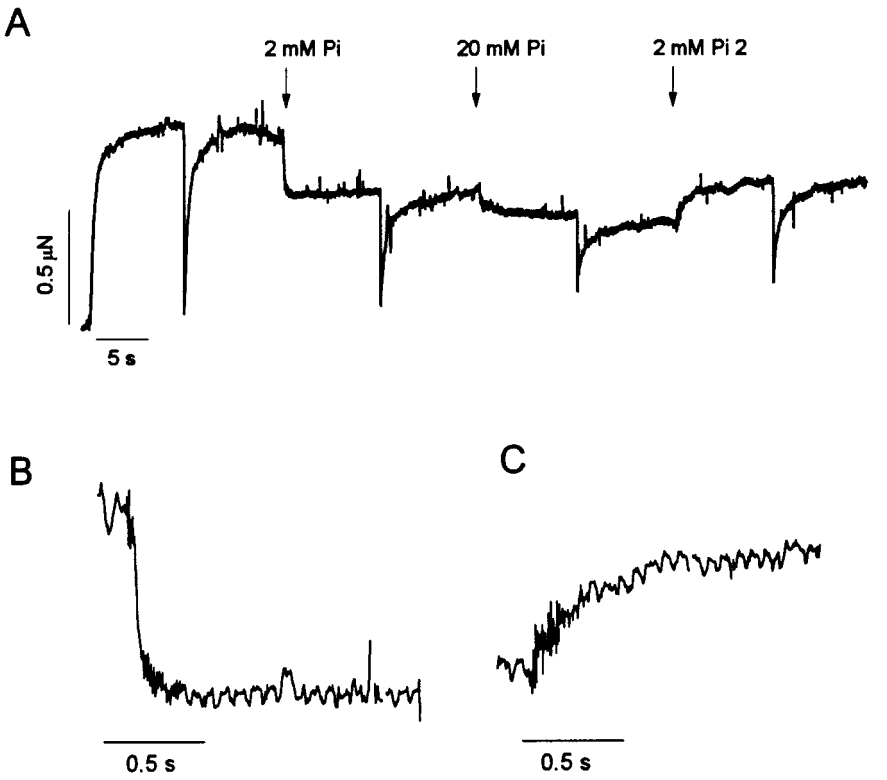
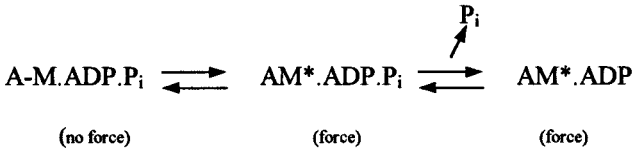


FIGURE 7 (*A*) To measure $k_{Pi(+)}$, $k_{Pi(-)}$, and k_{TR} at the same final $[P_i]$ during a single activation-relaxation cycle, a rabbit soleus myofibril activated in P_i-free solution was subjected to a series of jumps in $[P_i]$ (20°C). (*B*) Expanded force transient initiated by increasing $[P_i]$ to 2 mM. (*C*) Expanded force transient initiated by decreasing $[P_i]$ to 2 mM, starting from 20 mM P_i. 2 mM P_i: $k_{Pi(+)} = 14\ s^{-1}$; $k_{Pi(-)} = 3.1\ s^{-1}$; $k_{TR} = 2.8\ s^{-1}$. $l_o = 82\ \mu m$; *s.l.* = 2.6 μm.

et al., 1992) to take into account the difference in temperature, there is a good agreement between our data for myofibrils and those from caged phosphate experiments in skinned fibers. This conclusion can also be extended to the comparison of $k_{Pi(+)}$ estimated in myofibrils from P_i jumps and the values of the P_i-sensitive rate constant ($2\pi b$) observed at the same $[P_i]$ by sinusoidal analysis in skinned fibers (Kawai and Halvorson, 1991). For skinned fibers (see review by Morris and Homsher, 1998), as for rabbit psoas myofibrils, $k_{Pi(+)}$ was found to be three to four times higher than the rate of force redevelopment (k_{TR}) measured under the same conditions.

For rabbit psoas myofibrils, as previously observed for skinned fibers with caged P_i (Dantzig et al., 1992; Walker et al., 1992), the kinetics of the transient decrease in force induced by a sudden increase in $[P_i]$ strongly depended on the final ligand concentration, being $k_{Pi(+)}$ 7.4 ± 1.0 s^{-1} and 40 ± 8 s^{-1} at 1 mM and 8 mM P_i, respectively ($n = 10$; preliminary experiments at 5°C not shown). In the $[P_i]$ range we tested (1–10 mM), no tendency of the kinetics of the force decay transient to saturate with the raising of $[P_i]$ was observed, and, because of time resolution limitations of our present experimental setup, we could not resolve $k_{Pi(+)}$ at $[P_i]$ higher than 8 mM. Moreover, timing uncertainties of the start of the solution change (see Materials and Methods) made it impossible to resolve the lag phase described in skinned fibers after photolysis of caged P_i (Dantzig et al., 1992). In these studies, where the time resolution is higher than ours, a hyperbolic relation between $k_{Pi(+)}$ and final $[P_i]$ is observed (Dantzig et al., 1992; Walker et al., 1992). This indicates that P_i binding is a two-step process: a fast binding equilibrium followed by a slower isomerization. The latter is responsible for the force generation power stroke (for a review see Morris and Homsher, 1998):



Scheme 1.

Interestingly, Geeves and collaborators, using skinned rabbit psoas fibers (Fortune *et al.,* 1991, 1994), arrive at the same conclusion from an analysis of the effect of P_i on force transients induced by hydrostatic pressure jumps. More recently, Ranatunga (1999) proposed a three-step P_i release mechanism to accommodate observations from pressure and temperature jump experiments. We are unable to discriminate between chemomechanical coupling schemes that associate the power stroke with the P_i release or a preceding isomerization.

The kinetics of the transient decline in force observed when $[P_i]$ was suddenly increased in our P_i jump and in caged P_i experiments are similar. Taking the linear relation

observed in myofibrils between $k_{Pi(+)}$ and final [P_i] (data at 1 and 8 mM P_i are given above; data at 5 mM P_i are shown in Table 1) as the initial part of a hyperbola, the intercept ($4.2 \pm 2.3\ s^{-1}$), corresponding to the forward rate of the force-generating step, and the slope ($3.0 \pm 0.9\ 10^3\ M^{-1}s^{-1}$), corresponding to the second-order binding constant for P_i, are both in reasonable agreement with previous observations in skinned fibers, with caged P_i experiments (Dantzig et al., 1992; Walker et al., 1992), or sinusoidal length perturbations in the presence of P_i (Kawai and Halvorson, 1991).

The kinetics of the transient rise in force ($k_{Pi(-)}$) initiated in isometrically contracting psoas myofibrils by a sudden decrease in [P_i] is three to four times slower than $k_{Pi(+)}$ and not significantly different from k_{TR} measured at the same final [P_i]. This result is unexpected from a simple P_i release pathway such as Scheme 1 or from those models that introduce Ca-sensitive transitions preceding the power-stroke to account for the fact that P_i transients are faster than k_{TR} and show little or no calcium sensitivity (Millar and Homsher, 1992).

The marked asymmetry of the rate of force change when a given final [P_i] is approached from a higher versus a lower [P_i] could be due to artifacts of our solution change method and/or to lack of control of mechanical conditions of contraction in myofibrils. To control for such artifacts we examined force responses produced by sudden changes in calcium or MgADP concentrations. If such artifacts exist, asymmetries similar to those of P_i jumps should show up.

Calcium jump experiments (results not shown) were performed by first activating myofibrils at intermediate pCa, followed by jumps of pCa to full activation and back. The kinetics of force rise and decline induced by a jump increase in [Ca^{2+}] and by its reversal were essentially similar. An asymmetry, if present, was opposite from that for P_i jumps (i.e., the transient rise in force was as fast or even faster than the transient force decline at the same final pCa; Poggesi et al., unpublished results).

A similar test was performed, subjecting rabbit psoas myofibrils to jumps in [MgADP] from the contaminant value (probably less than 5 μM) to 3 mM and back (Fig. 8). As previously observed in skinned rabbit psoas fibers, the presence of a millimolar concentration of MgADP in activating solutions (Cooke and Pate, 1985) or its sudden increase after photolysis of caged ADP (Lacktis and Homsher, 1987; Lu et al., 1993) increases isometric force development and slows down k_{TR}, by ~20%. Our experiments confirm these observations. In myofibrils subjected to MgADP jumps, isometric force and k_{TR} in 3 mM MgADP are 1.18 ± 0.02 and 0.77 ± 0.03 of control values ($n = 15$), and the rates of force changes induced by sudden changes in [MgADP] are not significantly different from k_{TR} measured just after the jumps, independent of the direction of the change in ligand concentration and independent of its effect on force.

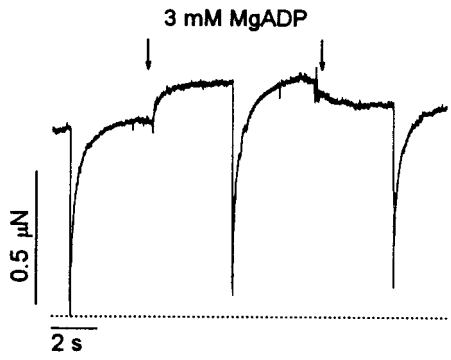


FIGURE 8 Force changes in a rabbit psoas myofibril (5°C) following sudden changes in [MgADP] from contaminant to 3 mM and back. The force rise and decline are symmetrical: $k_{ADP} = 1.8\ s^{-1}$ (for both jumps); k_{TR} is 2.6 and 2.0 s^{-1} in control and 3 mM MgADP conditions, respectively. Force in 3 mM MgADP is 1.24 the control. Dotted line: force baseline. $l_o = 74\ \mu m$; *s.l.* = 2.5.

These control experiments are evidence that the asymmetry in the values estimated for rabbit psoas myofibrils from declining or rising force transients did not arise from systematic artifacts of our method, but we did not test for all mechanical artifacts. A certain degree of internal shortening during contraction is expected in our experiments, and this would be amplified by sarcomere inhomogeneity. We do not think that sarcomere inhomogeneity constitutes a major source of artifact because the series compliance of rabbit psoas myofibrils estimated in a previous work is rather small (~5% l_o; Tesi et al., 1999). Moreover, if force changes are mainly governed by intersarcomere dynamics, we expect the kinetics of P_i jumps as measured in myofibrils to be barely P_i-dependent, as is shortening velocity (Cooke and Pate, 1985). Clearly this is not the case, as the effect of final [P_i] on the kinetics of P_i transients is strong.

The analysis of MgADP jumps and of the effects of calcium on the kinetics of P_i jumps and the experiments performed with rabbit soleus myofibrils all argue that the kinetics of the transient rise in force initiated by a sudden decrease in [P_i] and k_{TR} are very close. $k_{Pi(-)}$ and k_{TR} are shown to have not only the same value but also the same calcium, phosphate, and temperature dependencies, which are different from those of $k_{Pi(+)}$. Experiments performed at 20°C in rabbit soleus myofibrils (Table 1), i.e., in conditions where the time resolution of P_i transients was less critical, confirmed the presence of marked asymmetry in the P_i jumps. It is interesting to note that in soleus myofibrils at 15°C, in conditions where presumably the P_i release steps are not kinetically isolated from the rest of the reaction pattern and therefore could contribute to the overall rate-limiting step (Millar and Homsher, 1992), the asymmetry is less evident and the differences between $k_{Pi(+)}$, $k_{Pi(-)}$, and k_{TR} are greatly reduced.

We conclude that the asymmetrical behavior in the kinetics of force changes initiated by an increase or a decrease in $[P_i]$ reflects intrinsic properties of the cross-bridge cycle, when P_i release is not rate limiting for force generation. Attempts to fit P_i jumps with current models of cross-bridge action failed, suggesting a possible role for thin filament activation dynamics induced by cross-bridge detachment or by shifts in the ratio of weak to strong attached states at different $[P_i]$.

The authors gratefully acknowledge Dr. Earl Homsher for helpful discussion and for the gift of the software kfit/ksim written by Dr. Neil Millar; Dr. Phil W. Brandt for the computing of solution composition and helpful comments on the manuscript; Dr. Jacopo Chini for enzyme immobilization and help with the P_i-scavenging protocol; and Mr. Alessandro Aiazzi, Mr. Mario Dolfi, and Mr. Adrio Vannucchi for technical assistance.

This work was supported by the Ministero dell'Università e della Ricerca scientifica e Tecnologica (40%) and the Università degli studi di Firenze (60%). The financial support of the EU is also acknowledged.

REFERENCES

Baginski, E. S., P. P. Foa, and B. Zak. 1967. Determination of phosphate: study of labile organic phosphate interference. *Clin. Chim. Acta.* 15: 155–158.

Barman, T., M. Brune, C. Lionne, N. Piroddi, C. Poggesi, R. Sthele, C. Tesi, F. Travers, and M. Webb. 1998. ATPase and shortening rates in frog fast skeletal myofibrils by time-resolved measurements of protein-bound and free P_i. *Biophys. J.* 74:3120–3130.

Bartoo, M. L., V. I. Popov, L. A. Fearn, and G. H. Pollack. 1993. Active tension generation in isolated skeletal myofibrils. *J. Muscle Res. Cell Motil.* 14:498–510.

Bowater, R., and J. Sleep. 1988. Demembranated muscle fibers catalyze a more rapid exchange between phosphate and adenosine triphosphate than actomyosin subfragment 1. *Biochemistry.* 27:5314–5323.

Brandt, P. W., F. Colomo, N. Piroddi, C. Poggesi, and C. Tesi. 1998. Force regulation by Ca^{2+} in skinned cardiac myocytes of frog. *Biophys. J.* 74:1994–2004.

Brandt, P. W., R. N. Cox, M. Kawai, and T. Robinson. 1982. Effect of cross-bridge kinetics on apparent Ca^{2+} sensitivity. *J. Gen. Physiol.* 79:997–1016.

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proc. Natl. Acad. Sci. USA.* 83:3265–3269.

Brenner, B. 1990. Muscle mechanics and biochemical kinetics. *In* Molecular Mechanism and Biochemical Kinetics. J. M. Squire, editor. McMillan Press, London. 77–149.

Brune, M., J. L. Hunter, J. E. T. Corrie, and M. R. Webb. 1994. Direct, real time measurements of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment1 ATPase. *Biochemistry.* 33:8262–8271.

Chase, P. B., and M. J. Kushmerick. 1988. Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophys. J.* 53:935–946.

Colomo, F., S. Nencini, N. Piroddi, C. Poggesi, and C. Tesi. 1998. Calcium dependence of the apparent rate of force generation in single myofibrils from striated muscle activated by rapid solution changes. *Adv. Exp. Med. Biol.* 453:373–382.

Colomo, F., N. Piroddi, C. Poggesi, G. te Kronnie, and C. Tesi. 1997. Active and passive forces of isolated myofibrils from cardiac and fast skeletal muscle of the frog. *J. Physiol. (Lond.).* 500:535–548.

Cooke, R. 1997. Actomyosin interactions in striated muscle. *Physiol. Rev.* 77:671–697.

Cooke, R., K. Franks, G. B. Luciani, and E. Pate. 1988. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J. Physiol. (Lond.).* 395:77–97.

Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys. J.* 48:789–798.

Dantzig, J. A., Y. E. Goldman, N. C. Millar, W. Lacktis, and E. Homsher. 1992. Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibers. *J. Physiol. (Lond.).* 451:247–278.

Eisenberg, E., T. L. Hill, and Y. Chen. 1980. Cross-bridge model of muscle contraction. *Biophys. J.* 29:195–227.

Fortune, N. S., M. A. Geeves, and K. W. Ranatunga. 1991. Tension responses to rapid pressure release in glycerinated rabbit muscle fibers. *Proc. Natl. Acad. Sci. USA.* 88:7323–7327.

Fortune, N. S., M. A. Geeves, and K. W. Ranatunga. 1994. Contractile activation and force generation in skinned rabbit muscle fibers: effect of hydrostatic pressure. *J. Physiol. (Lond.).* 474:283–290.

Friedman, A. L., and Y. E. Goldman. 1996. Force and force transients in bundles of 1–3 myofibrils from rabbit psoas muscle. *Biophys. J.* 71: 2774–2785.

Geeves, M. A. 1991. The dynamics of actin and myosin association and the cross-bridge model of muscle contraction. *Biochem. J.* 274:1–14.

Geeves, M. A., and K. C. Holmes. 1999. Structural mechanism of muscle contraction. *Annu. Rev. Biochem.* 68:687–728.

Goldman, Y. E. 1987. Kinetics of the actomyosin ATPase in muscle fibers. *Annu. Rev. Physiol.* 49:637–654.

Hibberd, M. G., J. A. Dantzig, D. R. Trentham, and Y. E. Goldman. 1985. Phosphate release and force generation in skeletal muscle fibers. *Science.* 228:1317–1319.

Hibberd, M. G., and D. R. Trentham. 1986. Relationships between chemical and biochemical events during muscular contraction. *Annu. Rev. Biophys. Biophys. Chem.* 15:119–161.

Homsher, E., J. Lacktis, and M. Regnier. 1997. Strain-dependent modulation of phosphate transients in rabbit skeletal muscle fibers. *Biophys. J.* 72:1780–1791.

Kawai, M. 1986. The role of orthophosphate in crossbridge kinetics in chemically skinned rabbit psoas fibers as detected with sinusoidal and step length alterations. *J. Muscle Res. Cell Motil.* 7:421–434.

Kawai, M., K. Güth, K. Winnikes, C. Haist, and J. C. Rüegg. 1987. The effect of inorganic phosphate on the ATP hydrolysis rate and the tension transients in chemically skinned rabbit psoas fibers. *Pflügers Arch.* 408:1–9.

Kawai, M., and H. R. Halvorson. 1991. Two step mechanism of phosphate release and the mechanism of force generation in chemically skinned fibers of rabbit psoas muscle. *Biophys. J.* 59:329–342.

Lacktis, J. W., and E. Homsher. 1987. The force response to photogeneration of ADP in isometrically contracting glycerinated rabbit psoas muscle fibers. *Biophys. J.* 51:475a (Abstr.).

Lu, Z., R. L. Moss, and J. W. Walker. 1993. Tension transients initiated by photogeneration of MgADP in skinned skeletal muscle fibers. *J. Gen. Physiol.* 101:867–888.

Martyn, D. A., and A. M. Gordon. 1992. Force and stiffness in glycerinated rabbit psoas fibers. *J. Gen. Physiol.* 99:795–816.

Metzger, J., and R. Moss. 1990. Calcium-sensitive cross-bridge transition in mammalian fast and slow skeletal muscle fibers. *Science.* 247: 1088–1090.

Millar, N. C., and E. Homsher. 1990. The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibers. *J. Biol. Chem.* 265:20234–20240.

Millar, N. C., and E. Homsher. 1992. Kinetics of force generation and phosphate release in skinned rabbit soleus muscle fibers. *Am. J. Physiol.* 262:C1239–C1245.

Morris, C., and E. Homsher. 1998. The use of caged compounds. *In* Current Methods in Muscle Physiology: Advantages, Problems, and Limitations. H. Sugi, editor. Oxford University Press, Oxford. 71–89.

Nencini, S., N. Piroddi, F. Colomo, C. Tesi, and C. Poggesi. 1999. Relaxation rate of single skeletal muscle myofibrils following rapid solution change. *Biophys. J.* 76:163a (Abstr.).

Nosek, T. M., K. Y. Fender, and R. E. Godt. 1987. It is diprotonated inorganic phosphate that depresses force in skinned skeletal muscle fibers. *Science.* 236:191–193.

Pate, E., and R. Cooke. 1989a. Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke. *Pflügers Arch.* 414:73–81.

Pate, E., and R. Cooke. 1989b. A model of cross-bridge action: the effects of ATP, ADP and P_i. *J. Muscle Res. Cell Motil.* 10:181–196.

Pate, E., K. Franks-Skiba, and R. Cooke. 1998. Depletion of phosphate in active muscle fibers probes actomyosin states within the powerstroke. *Biophys. J.* 74:369–380.

Piroddi, N., S. Nencini, F. Colomo, C. Poggesi, and C. Tesi. 1999. The effect of phosphate on force generation in single myofibrils from rabbit skeletal muscle. *Biophys. J.* 76:269a (Abstr.).

Ranatunga, K.W. 1999. Effects of inorganic phosphate on endothermic force generation in muscle. *Proc. R. Soc. Lond. B.* 266:1381–1385.

Regnier, M., and E. Homsher. 1998. The effect of ATP analogs on posthydrolytic and force development steps in skinned skeletal muscle fibers. *Biophys. J.* 74:3059–3071.

Regnier, M., D. M. Lee, and E. Homsher. 1998. ATP analogs and muscle contraction: mechanics and kinetics of nucleoside triphosphate binding and hydrolysis. *Biophys. J.* 74:3044–3058.

Regnier, M., C. Morris, and E. Homsher. 1995. Regulation of cross-bridge transition from a weakly to strongly bound state in skinned rabbit muscle fibers. *Am. J. Physiol.* 38:C1532–C1539.

Sleep, J., and R. Hutton. 1980. Exchange between inorganic P_i and ATP in the medium by actomyosin subfragment-1. *Biochemistry.* 19: 1276–1283.

Stienen, G. J. M., M. C. M. Roosemalen, M. G. A. Wilson, and G. Elzinga. 1990. Depression of force by phosphate in skinned muscle fibers of the frog. *Am. J. Physiol.* 259:C349–C357.

Tesi, C., F. Colomo, S. Nencini, N. Piroddi, and C. Poggesi. 1999. Modulation by substrate concentration of maximal shortening velocity and isometric force in single myofibrils from frog and rabbit fast skeletal muscle. *J. Physiol. (Lond.).* 516:847–853.

Wahr, P. A., H. C. Cantor, and J. M. Metzger. 1997. Nucleotide-dependent contractile properties of Ca^{2+}-activated fast and slow skeletal muscle fibers. *Biophys. J.* 72:822–834.

Walker, J. W., Z. Lu, and R. L. Moss. 1992. Effects of Ca^{2+} on the kinetics of phosphate release in skeletal muscle. *J. Biol. Chem.* 267:2459–2466.

Wang, G., and M. Kawai. 1997. Force generation and phosphate release steps in skinned rabbit soleus slow-twitch muscle fibers. *Biophys. J.* 73:878–894.

Webb, M. R., M. G. Hibberd, Y. E. Goldman, and D. R. Trentham. 1986. Oxygen exchange between P_i in the medium and water during ATP hydrolysis mediated by skinned fibers from rabbit skeletal muscle. *J. Biol. Chem.* 261:15557–15564.

White, H. D., and E. W. Taylor. 1976. Energetics and mechanism of actomyosin ATPase. *Biochemistry.* 15:5818–5826.

Wilson, G. J., S. E. Shull, and R. Cooke. 1995. Inhibition of muscle force by vanadate. *Biophys. J.* 68:216–226.