At Physiological Temperatures the ATPase Rates of Shortening Soleus and Psoas Myofibrils Are Similar

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ABSTRACT We obtained the temperature dependences of the adenosine triphosphatase (ATPase) activities (calcium-activated and relaxed) of myofibrils from a slow muscle, which we compared with those from a fast muscle. We chose rabbit soleus and psoas because their myosin heavy chains are almost pure: isoforms I and IIX, respectively. The Arrhenius plots of the ATPases are linear (4–35°C) with energies of activation for soleus myofibrils 155 kJ mol^{-1} (activated) and 78 kJ mol^{-1} (relaxed). With psoas myofibrils, the energies of activation were 71 kJ mol^{-1} (activated) and 60 kJ mol^{-1} (relaxed). When extrapolated to 42°C the ATPase rates of the two types of myofibril were identical: 50 s^{-1} (activated) and 0.23 s^{-1} (relaxed). Whereas with psoas myofibrils the K_m for adenosine triphosphate (activated ATPase) is relatively insensitive to temperature, that for soleus myofibrils increased from 0.3 μM at 4°C to 66.5 μM at 35°C. Our results illustrate the importance of temperature when comparing the mechanochemical coupling in different types of muscle. We discuss the problem of how to reconcile the similarity of the myofibrillar ATPase rates at physiological temperatures with their different mechanical properties.

INTRODUCTION

The mechanochemical coupling in muscle contraction relies on specific interactions of the different myosin head adenosine triphosphatase (ATPase) intermediates with the actin filaments and associated conformational changes of the heads. To fully understand this coupling, one should measure the chemical kinetics and mechanical properties of muscle fibers under identical conditions, ideally simultaneously. We are attempting to do this with myofibrils as the experimental material. Myofibrils are the functionally contractile units of muscle and yet they are small enough for study by rapid reaction methods. Both chemical and mechanical kinetic studies have been carried out with myofibrils (Lionne et al., 2002, and references cited therein).

In previous works, we studied the mechanochemical coupling in rabbit psoas myofibrils (Lionne et al., 1996, 1999, 2003). Now, this is a fast twitch muscle that contains 92% of the isoform myosin heavy chain, MHC-IIX (Tikunov et al., 2001) and it seemed important to extend these studies to a slow twitch muscle with a fundamental question: which step(s) on the myofibrillar ATPase reaction pathway determines whether the muscle is ''fast'' or ''slow''? Further, from the works of Bottinelli et al. (1996), Wang and Kawai (2001), and Ranatunga (1982, 1984), it appears that certain of the mechanical properties of slow twitch muscle fibers (soleus) are more temperature sensitive than are those from fast twitch fibers (psoas). Do the ATPases of myofibrils from slow and fast twitch muscles also have different temperature sensitivities?

Here we studied the chemical kinetics of a slow twitch muscle, rabbit soleus. We chose this muscle because its myosin heavy chain is virtually pure at 97% MHC-I (Tikunov et al., 2001). As our method for preparing myofibrils from psoas fibers (Herrmann et al., 1993) could not be used with the soleus, we modify this procedure based on the works of Tesi et al. (2000). Both methods (standard for psoas and modified for soleus) involved treatment with Triton X-100 and subsequent washing to remove the fiber membranes (in particular the sarcoplasmic reticulum) with their associated ATPase activities.

We measured the steady-state ATPase parameters (k_{cat}, K_m for adenosine triphosphate (ATP)) of rabbit soleus myofibrils under two mechanical conditions, ''relaxing'' (low Ca^{2+}) and ''unloaded shortening'' (0.1 mM Ca^{2+}). We investigated the dependences of the parameters on temperature in the range 4–35°C with three objectives: first, to obtain estimates of the parameters at physiological temperatures (soleus myofibrils are difficult to handle at temperatures above 35°C); second, to compare these dependences with those of the ATPases of psoas myofibrils; finally, to obtain mechanistic information, which can be done when there are ''breaks'' or curvatures in the temperature dependence of rate constants (Arrhenius plots) (Biosca et al., 1984; Lionne et al., 1999).

We show here that the Ca^{2+}-activated steady-state ATPase parameters with soleus myofibrils are considerably more sensitive to temperature than those measured with relaxed soleus myofibrils and, in particular, with psoas myofibrils, both in the relaxed and activated conditions. We also show that, when extrapolated to the physiological temperatures of the rabbit, the ATPase steady-state rates of slow myofibrils (soleus) are similar to those of fast

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myofibrils (psoas), both under relaxing conditions and during unloaded shortening.

MATERIALS AND METHODS

Myofibrils and chemicals

Rabbit psoas muscle myofibrils were prepared as described in Herrmann et al. (1993) and stored at 4°C for up to 3 days in a storage buffer (50 mM Tris, 100 mM potassium acetate, 5 mM KCl, 2 mM magnesium acetate, 2 mM DTT, 0.5 mM sodium azide, 0.2 mM PMSF, 10 μM leupeptin, and 5 μM pepstatin, adjusted to pH 7.4 at room temperature with acetic acid).

When prepared following this procedure, myofibrils from rabbit soleus muscles were inhomogeneous in sarcomere lengths (some myofibrils shortened) and often aggregated. Therefore, these myofibrils were prepared following a procedure that is a modification of that described by Tesi et al. (2000). Briefly, soleus muscles were isolated and tied to wooden sticks, close to resting length. Deep cuts were carried out longitudinally with a scalpel to allow efficient solution diffusion inside the muscles. To ensure ATP depletion, the muscles were stored at 0–4°C for 2 days in Ringer-EGTA solution (50 mM Tris, 1 mM EGTA, 100 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.5 mM sodium azide, 0.2 mM PMSF, 10 μM leupeptin, and 5 μM pepstatin, adjusted to pH 7.0 with HCl). They were then transferred into a rigor solution (50 mM Tris, 1 mM EGTA, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.5 mM sodium azide, 0.2 mM PMSF, 10 μM leupeptin, and 5 μM pepstatin, adjusted to pH 7.0 with HCl) at 0–4°C for 24 h and then into rigor solution containing 50% glycerol for another 24 h. Finally, the soleus muscles were stored at –20°C in the same rigor solution plus 50% glycerol for at least 48 h and used within 7–8 weeks. Just before the preparation of myofibrils, tendons and connective tissue were carefully removed with a scalpel without stretching the fibers.

The rest of the preparation was similar to the standard procedure used for preparing psoas myofibrils (Herrmann et al., 1993). To remove the fiber membranes (especially the sarcoplasmic reticulum), the fibers were homogenized in the homogenization buffer which is identical to the storage buffer (see above) plus 0.5% Triton X-100 and 5 mM EDTA but without magnesium acetate. The homogenization procedure, using a Sorval Omnimix at maximum speed, was shorter with soleus (2×10 s) than with psoas (6×10 s). A 60-s resting time between each homogenization was necessary to prevent warming up of the solutions. After preparation, the myofibrillar suspensions were used within 1 day.

Immediately before experimentation, the myofibrils were washed twice (centrifugation 10 min at $2000 \times g$) in 50 mM Tris-acetate, pH 7.4, 100 mM K-acetate, 5 mM KCl and either 0.1 mM $CaCl_2$, 2 mM Mg-acetate (activating buffer, $+Ca^{2+}$), or 2 mM EGTA, 5 mM Mg-acetate (relaxing buffer, $-Ca^{2+}$). Any aggregates were removed by filtration through a polypropylene filter of 149-μm pore openings (Spectra Mesh, Spectrum Medical Industries, Laguna Hills, CA). The concentration of myosin heads in the myofibrillar suspension was measured by absorption at 280 nm, assuming that in soleus myofibrils, the molar extinction coefficient and percentage of myosin are the same as in psoas myofibrils (Herrmann et al., 1994).

Sarcomere lengths were measured by transmitted light microscopy using a DMR B Nomarski microscope (Leica, Wetzlar, Germany), tube factor 1.6×, with a PL APO 63× or 100× immersion oil objective (NA 1.40). The images thus obtained were captured with a MicroMax (Unanderra, Australia)1300 Y/HS (B/W) cooled (−10°C) CCD camera as 8-bit images (C mount 1×) and MetaMorph (v.4.6r5) controller program (RS Princeton Instruments, Trenton, NJ) run by a PC compatible microcomputer. The images were saved as TIFF 8-bit format. Sarcomere lengths were measured with Matrox Inspector 2.2 image processing software (Matrox Electronic Systems, Montreal, Canada). For each experimental condition, 10–20 myofibrils were analyzed and their sarcomere lengths averaged.

Apyrase (200 units/mg), creatine kinase (300 units/mg), phosphocreatine, cyclopiazonic acid (CPA), and ouabain were from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France).

Testing soleus myofibrils for endogenous adenosine diphosphate (ADP)

Contaminating ADP (or less probably ATP) was tested for by treating the myofibrils with apyrase (both ADP and ATP cleaved to adenosine monophosphate plus P_i) and then estimating any P_i by the fluorescent phosphate binding protein (MDCC-PBP) of Brune et al. (1994). Typically, reaction mixtures contained 1 μM myofibrils (as myosin heads) in relaxing buffer, 10 μM MDCC-PBP and a P_i-mop system that removes any contaminant traces of P_i (Brune et al., 1994). The fluorescence signal recorded in a FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex, Lille, France) with excitation wavelength 436 nm, emission wavelength 470 nm, and slit widths of 1 nm. This was followed by the addition of apyrase (0.7 units/ml) and the fluorescence signal was again recorded. Calibration was carried out by the addition of ADP (to 1 μM) and recording the increase in fluorescence. A baseline (negative control) was carried out following the same procedure but without myofibrils. This baseline was then subtracted from the signal measured with myofibrils, a procedure that allowed eliminating artifacts due to 1), photobleaching of the MDCC-PBP fluorescence, and 2), contaminant P_i present with the apyrase.

Kinetic studies

These were carried out in a home-built, thermostatically controlled, rapid flow quench apparatus (Barman and Travers, 1985). The procedure was to mix myofibrils with [γ-^{32}P]ATP in the apparatus. The reaction mixtures were quenched at different times in acid (22% TCA, 1 mM KH_2PO_4) and the total P_i concentrations determined by the filter paper method of Reimann and Umfleet (1978). This type of experiment allows the measurement of the kinetics of formation of total P_i, i.e., free P_i plus myosin head bound P_i. The data were fitted using linear or nonlinear regression (GraFit version 3.03, Erithacus Software, Staines, UK). For statistical analysis, SigmaStat version 1.0 (Jandel, San Rafael, CA) was used. A two-way analysis of variance was used to test the difference in the energy of activation between soleus and psoas ATPases. For the other kinetic parameters, to assess differences between soleus and psoas, a Student's t-test was used. Statistical significance was accepted at a probability level (p) < 0.05. The $\pm$ values refer to standard deviations of the data. We have already discussed the problems of the quality of fits and errors (Lionne et al., 1999).

RESULTS

Quality control of soleus myofibrils

Our standard protocol for preparing rabbit psoas myofibrils could not be applied to the soleus because it gave a significant proportion of shortened and aggregated myofibrils. Therefore, we modified this procedure, based on the protocol of Tesi et al. (2000), as described in Materials and Methods. We checked on possible perturbations of the ATPases of psoas myofibrils when prepared with this modified procedure. Then, we checked on the overall structure of soleus myofibrils, their contractility in the presence of ATP, and their thermal stability.

Possible perturbations of the psoas myofibrillar ATPase with the modified protocol

To check possible perturbations induced by the modification of the standard myofibril preparation procedure, we prepared psoas myofibrils by the modified procedure and compared

their Ca^{2+}-activated ATPase activity with that of myofibrils prepared by the original method. The two ATPase progress curves were virtually identical at both 4 and 20°C (data not illustrated). Therefore, we concluded that, with psoas myofibrils as control, the new preparation procedure does not affect myofibrillar ATPase activities.

Microscopic structure of soleus myofibrils

A good preparation should contain myofibrils that are as straight as possible and with sarcomeres that are homogeneous in length. The presence of large fiber bundles (containing >10 myofibrils) should be avoided to eliminate possible diffusion problems. We previously checked the quality of psoas myofibril preparations (Herrmann et al., 1993). As observed under the optical microscope, myofibrils prepared from soleus rabbit muscles with our modified procedure were straight and either discrete or, as shown in Fig. 1 *A*, grouped into thin bundles of 2–4 of diameter <2 μm. They were 10–25 sarcomeres long and the sarcomere lengths were homogeneous both within single myofibrils and between different myofibrils at 2.4 ± 0.1 μm. This corresponds to resting length and 100% filament overlap (Woledge et al., 1985). The preparations appeared to be free of sarcoplasmic reticulum vesicles.

As shown in Fig. 1 *B*, when incubated with ATP, soleus myofibrils shortened in the presence of Ca^{2+} and, because they were not anchored, like psoas myofibrils, they finished by ''overcontracting'' to give aggregates. Usually, overcontracted myofibrils occurred in larger aggregates (>50 μm wide) than shown in Fig. 1 *B*, suggesting the presence of attractive forces between individual myofibrils during or after the shortening process. As shown in Fig. 1 *C*, there was no shortening in the absence of Ca^{2+} (mean sarcomere lengths were 2.4 ± 0.1 μm in the presence or in the absence of ATP), which suggests that the Ca^{2+} regulatory system was well preserved.

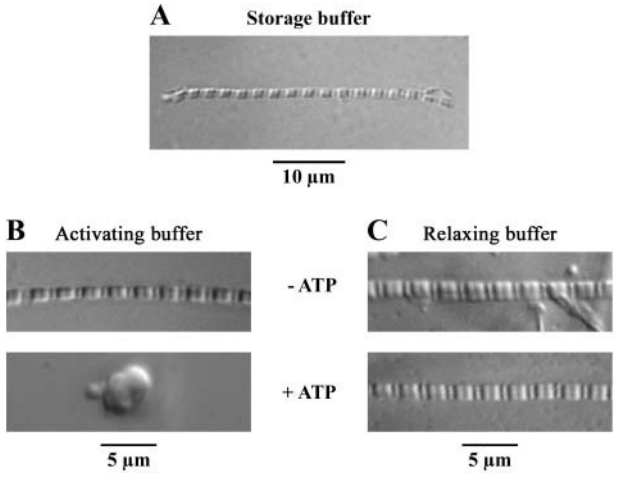

FIGURE 1 (*A*) Image of a bundle of 3–4 soleus myofibrils in storage buffer obtained with a Nomarski microscope using objective 63×. (*B* and *C*) Images of four different soleus myofibrils using objective 100× in activating buffer (*B*, $+Ca^{2+}$) or in relaxing buffer (*C*, $-Ca^{2+}$) in the absence (*top panels*) or the presence (*bottom panels*) of 100 μM ATP at room temperature (22–25°C). At the time the pictures were taken (several minutes after addition of ATP), all the ATP had been hydrolyzed. The sarcomere lengths of the myofibrils shown were 2.40 μm (*A*), 2.34 μm (*B*, *top*), 2.45 μm (*C*, *top*), and 2.41 μm (*C*, *bottom*). Experimental details are in Materials and Methods.

Thermal stability of soleus myofibrils

We checked the thermal stability of soleus myofibrils by incubating them for 1 h at 35°C in activating or relaxing buffer before measuring their ATPase activities at 25°C. The preincubation had little effect: activated steady-state rates were 1.70 ± 0.11 s^{-1} before and 1.72 ± 0.14 s^{-1} after preincubation; relaxed steady-state rates were 0.041 ± 0.002 s^{-1} before or after. Furthermore, the overall myofibrillar structures observed under the microscope were not altered by preincubation at 35°C (results not illustrated).

However, at temperatures above 35°C soleus myofibrils tend to aggregate and their ATPases seem to be poorly regulated by Ca^{2+} (data not illustrated). This thermal effect was not observed with psoas myofibrils, which are apparently stable up to 42°C.

Kinetic parameters of activated and relaxed soleus myofibrillar ATPases at 4 and 25°C

Typical time courses for myofibrillar ATPases are shown in Fig. 2 at 25°C and 4°C and the kinetic constants determined by computer fitting are summarized in Table 1. At 25°C (Fig. 2 *A*), the time course (0.6–15 s) with relaxed myofibrils was biphasic: a transient burst phase of amplitude *A* (kinetics, k_{obs}, not obtained on the timescale used) followed by a steady-state phase of rate k_{ss}. On a longer timescale (several minutes), the steady-state rate remained constant until the depletion of the ATP (data not shown). With Ca^{2+}-activated myofibrils, the time course (0.2–15 s) consisted of three consecutive phases: a transient burst phase (kinetics, k_{obs}, not obtained on the timescale used), an apparently linear phase of rate k_F, and then a slower steady state of rate k_S. This profile is characteristic of shortening psoas myofibrils (Houadjeto et al., 1991; Lionne et al., 1996): with these, k_F was attributed to the ATPase of myofibrils that are shortening under zero external load, and k_S to that of myofibrils that are in a pseudoisometric condition (Harada et al., 1990). k_S is probably not physiologically relevant because in this time range the sarcomere lengths of the myofibrils are shorter than the length of the thick filaments (Lionne et al., 1996; Barman et al., 1998).

At 4°C, and on the same timescale (0.2–15 s), the end of the kinetics of the transient burst phases and the beginning of the steady states were observed for both activated and relaxed ATPases (Fig. 2 *B*). The burst amplitudes (*A*) of activated and relaxed time courses were similar, both at 4 and

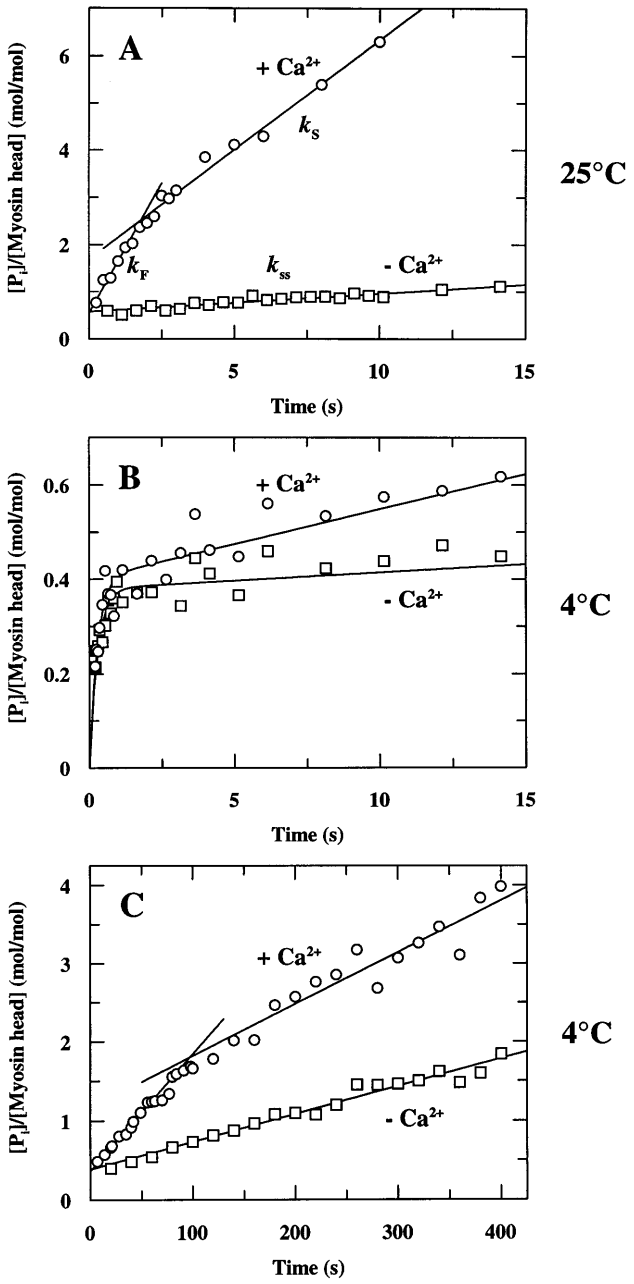

FIGURE 2 Time courses for the ATPase of Ca^{2+}-activated (○, $+Ca^{2+}$) or relaxed (□, $-Ca^{2+}$) soleus myofibrils at 25°C (*A*), and 4°C on two different time scales (*B*) and (*C*). Reaction mixtures were 5 μM myofibrils (as myosin heads) plus 50 μM [γ-^{32}P]ATP at 25°C, or 3 μM myosin heads in myofibrils plus 30 μM [γ-^{32}P]ATP at 4°C. The reaction mixtures were quenched at the times indicated and the total [^{32}P]P_i concentrations were determined.

25°C (Table 1). At 4°C, the kinetics of these burst phases (k_{obs}) were estimated and were also similar (Table 1, difference not significant). These similarities suggest that the kinetics of ATP binding and cleavage are insensitive to Ca^{2+}, presumably because, first, we start from rigor, and second, the cleavage step occurs on myosin detached from actin as with psoas myofibrils. Here, we did not investigate further these kinetics.

At 4°C, on a longer timescale (7–400 s), the characteristic shape of Ca^{2+}-activated myofibrillar ATPase was observed (Fig. 2 *C*): transient burst phase, linear phase of rate k_F, and slower steady state of rate k_S.

The kinetic parameters determined at 25 and 4°C are summarized in Table 1. The high temperature sensitivity of k_F and k_S relative to k_{ss} is noteworthy; it explains the important diminution (~7×) of the calcium activation factor ($F_{activ} = k_F/k_{ss}$) when the temperature was lowered from 25 to 4°C. In the following sections, the steady-state rate of Ca^{2+}-activated myofibrils is referred to as k_F.

Testing for sarcoplasmic reticulum Ca^{2+}- and Na^+-K^+-ATPases

Although we used 50% glycerol and 0.5% Triton X-100 to prepare the myofibrils, it was important to test for the absence of ATPase activities other than that of the myosin head. We did this by measuring the Ca^{2+}-activated myofibrillar ATPase (k_F) in the presence of the inhibitors cyclopiazonic acid (specific for sarcoplasmic reticulum Ca^{2+}-ATPase) or ouabain (specific for sarcolemma Na^+-K^+-ATPase) (Ebus and Stienen, 1996). The ATPase measurements were carried out as in Fig. 2 with reaction mixtures (5 μM myofibrils plus 50 μM [γ-^{32}P]ATP) containing no inhibitor, 10 μM CPA, or 1 mM ouabain. Two different batches of soleus myofibrils were tested, each series of kinetics being carried out on the same day. At 25°C, k_F was: no inhibitor, 1.22 and 1.10 s^{-1}; 10 μM CPA, 1.65 and 1.51 s^{-1}; and 1 mM ouabain, 1.10 and 1.12 s^{-1}.

We are unable to explain the activating effect of CPA on k_F, but we assume that the myofibrils were not contaminated with Ca^{2+}- and Na^+-K^+-ATPases. Kurebayashi and Ogawa (1991) have shown that 10 μM CPA induce a significant activation of the myofibrillar ATPase in submaximally Ca^{2+}-activated skinned fibers, but not in fully activated fibers. Here, the myofibrils were fully activated at 100 μM Ca^{2+} (see below).

Effect of ADP and P_i on the ATPase of Ca^{2+}-activated myofibrils (k_F)

Because the binding of ADP to soleus fibers is considered to be tight (e.g., Wang and Kawai, 1996; Shirakawa et al., 2000, and references cited therein), we tested the myofibrils for endogenous ADP. Thus, it could be that despite the washing procedure used in their preparation, the myofibrils still contained ADP. We checked on this by the use of apyrase (Sleep et al., 1994) and the MDCC-PBP of Brune et al. (1994) as described in Materials and Methods. On the addition of apyrase (0.7 units/ml) to 1 μM myofibrils plus 10 μM MDCC-PBP, there was a small increase in the fluorescence signal of 0.62 ± 0.2%. When 1 μM ADP was added to this mixture, there was a large increase of

TABLE 1 Kinetic parameters determined from the ATPase time courses in Fig. 2 at 25°C and 4°C

	$+Ca^{2+}$				$-Ca^{2+}$			
	A (mol/mol)	k_{obs} (s^{-1})	k_F (s^{-1})	k_S (s^{-1})	A (mol/mol)	k_{obs} (s^{-1})	k_{ss} (s^{-1})	F_{activ}
25°C	0.55	n.d.	1.10	0.46	0.58	n.d.	0.0385	28.6
4°C	0.40	~3.7	0.0148	0.0066	0.38	~3.6	0.0035	4.2
k at 25°C/k at 4°C	–	–	74	70	–	–	11	7

A and k_{obs}, amplitudes and rate constants of transient burst phases, respectively; F_{activ}, Ca^{2+}-activation factor as determined by the ratio of fast steady-state rate in activating condition (k_F) to steady-state rate in relaxing condition (k_{ss}).

75.8 ± 1.1%. We conclude that in our myofibrils <1% of the nucleotide sites are occupied by ADP.

We now checked on the effect of added ADP or P_i on k_F at 20°C (Table 2). As illustrated in Fig. 2, *A* and *C*, k_F appears to be linear up to a break (~2 s at 25°C and 100 s at 4°C) when there is a deceleration to k_S. This linearity suggests that the build-up of ADP and P_i during k_F, to ~10 μM at the break, has little effect on k_F. This was confirmed in experiments carried out with added 10 μM ADP and P_i either separately or together. Finally, we checked the effect of including the phosphocreatine/creatine kinase (PCr/CK) back-up system on k_F. This system is often used in muscle fiber work (e.g., He et al., 1998; Wang and Kawai, 2001) as it prevents the accumulation of ADP.

As shown in Table 2, neither added ADP, P_i, nor the PCr/CK system had any significant effect on k_F. We conclude that under our experimental conditions ADP and P_i up to at least 10 μM have no significant effects on k_F, the ATPase of actively shortening myofibrils.

Effect of temperature on the steady-state parameters of soleus myofibrillar ATPases

We now investigated the temperature sensitivities of the myofibrillar steady-state ATPases in more detail. As seen in Fig. 3 and in Table 3, k_F^{max} (k_F at saturating ATP concentrations) and K_m of k_F for ATP with Ca^{2+}-activated myofibrils are highly sensitive to the temperature, but the second order constant, k_F^{max}/K_m, is much less sensitive. We illustrate the temperature sensitivities of the steady-state parameters at two temperatures (16 and 30°C) in Fig. 3, *A* and *B*. At 35°C, k_F^{max} and K_m value determination was subject to large errors (see text above) but the estimates for both were significantly greater than at 30°C (Table 3). The dependence of K_m upon temperature (Fig. 3 *C*) gave a ΔG^o = 120 kJ mol^{-1}. Under relaxing conditions, the K_m for ATP was low, <1 μM at 4 and 30°C (results not illustrated).

TABLE 2 Effect of ADP and P_i on the ATPase of Ca^{2+}-activated myofibrils (k_F) at 20°C*

Additive	k_F (s^{-1})
None	0.50–0.70
10 μM ADP	0.73
10 μM P_i	0.60
10 μM ADP + 10 μM P_i	0.60
200 units/ml CK + 10 mM PCr	0.60
10 μM ADP + 200 units/ml CK + 10 mM PCr	0.66

*Reaction mixtures were 5 μM myofibrils plus 50 μM [γ-^{32}P]ATP in activating buffer with or without additive.

The temperature dependences (between 4 and 35°C) of the soleus myofibrillar ATPase rates at saturating concentrations of ATP are shown in Fig. 4 *A*. For comparative purposes, the dependences for psoas myofibrillar ATPases are shown in Fig. 4 *B* (Lionne et al., 1999). All the dependences could be fitted reasonably well to linear functions without breaks or curvatures. As summarized in Table 4, the energy of activation for the activated ATPase of soleus myofibrils is considerably larger ($p < 0.001$) than that of relaxed soleus myofibrils, but also of psoas myofibrils, both under activating and relaxing conditions. Whereas for the psoas ATPases both Arrhenius plots were parallel (Fig. 4 *B*; i.e., similar energies of activation for the relaxed and activated ATPases, $p > 0.05$), this was not the case for the soleus ATPases. This, as mentioned above, explains the temperature sensitivity of the activating factor, F_{activ}, in soleus myofibrils ($p < 0.001$).

A possible explanation for the high energy of activation of the k_F with soleus myofibrils is that the dissociation constant, K_d, for Ca^{2+} increases with a decrease in temperature. To check this, we compared the myofibrillar ATPase k_F at two concentrations of Ca^{2+}. At 4°C, k_F = 0.0194 s^{-1} and 0.0169 s^{-1} and at 25°C k_F = 1.44 s^{-1} and 1.45 s^{-1}, at 50 and 100 μM, respectively. The experiments were carried out as in Fig. 2. We conclude that at 100 μM Ca^{2+}, the myofibrillar ATPase is fully activated.

The extrapolated values of the ATPase rates to physiological temperatures (39–42°C) are shown in Table 4. In this range, the relaxed ATPase rates are almost identical for the two muscle types, and activated ATPase rates and Ca^{2+}-activating factors are similar. The physiological temperature of rabbit psoas and soleus could be lower than 39°C, but it increases during exercise. In either activated or relaxed conditions, the Arrhenius plots for psoas and soleus myofibrillar ATPases intersected at ~42°C, assuming that the plots are linear up to this temperature. Extrapolated values of k_F, k_{ss} and F_{activ} to 42°C are summarized in Table 4.

DISCUSSION

Here our aim was to compare and interpret the temperature dependences of the ATPases of myofibrils from rabbit skeletal

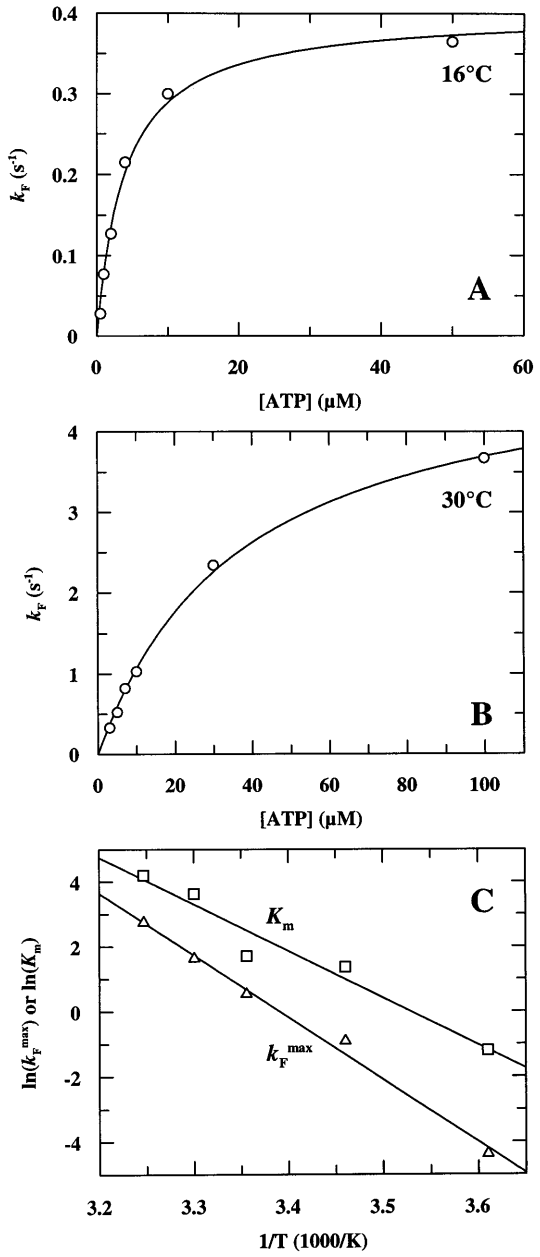

FIGURE 3 Dependence of Ca^{2+}-activated fast steady-state rate (k_F) of soleus myofibrillar ATPase upon the ATP concentration at 16°C (*A*) and 30°C (*B*). Each curve was fitted with a hyperbola function, the plateau giving k_F^{max}, the maximal fast steady-state rate extrapolated to infinite ATP concentration. The concentration of ATP at which the steady-state rate is half-maximal gives the K_m for ATP. (*C*) Temperature (T) dependences of k_F^{max} (*triangles*) and of K_m (*squares*) between 4 and 35°C.

muscles that are rich either in slow (soleus, 97% MHC-I) or in fast (psoas, 92% MCH-IIX) fibers. Our finding that at near physiological temperatures, the myofibrillar ATPases (whether Ca^{2+}-activated or not) of the two muscles are similar is surprising and we must interpret this in the context of the contractile process. But before we do so, we consider possible sources of artifacts and errors in interpretation.

TABLE 3 Effect of temperature on the steady-state parameters of the Ca^{2+}-activated ATPase of soleus myofibrils

Temperature (°C)	k_F^{max} (s^{-1})	K_m* (μM)	k_F^{max}/K_m ($\mu M^{-1}\ s^{-1}$)
4	0.0125 ± 0.0015	0.3 ± 0.3	~0.04
16	0.40 ± 0.02	3.9 ± 0.5	0.10
25	1.7 ± 0.1	5.5 ± 0.8	0.31
30	5.1 ± 0.2	37.5 ± 2.8	0.14
35	15.6 ± 4.9	66.5 ± 28.0	~0.23

*With reference to the fast ATPase rate, k_F, e.g., Fig. 2 *A*.

Validity of the ATPase measurements

First, does the mode of preparation affect the myofibrillar ATPases? We prepared psoas myofibrils by the modified (used here for soleus) as well as the standard (Herrmann et al., 1993) method and compared their ATPase activities (± Ca^{2+}): they were similar, both at 4 and 20°C. Therefore, we assume that the mode of preparation is unlikely to affect the ATPase activities of soleus myofibrils.

The slow ATP hydrolysis rate at 4°C (Fig. 2), together with an efficient ATP-regenerating system, may explain why, with soleus muscles, we could not use our standard myofibril preparation protocol which includes an ATP depletion step of 24 h (at ~4°C). When using this standard protocol with soleus, we get a significant proportion of shortened and aggregated myofibrils. Aggregation may be linked to shortening because over-contracted myofibrils are often found as large aggregates. It appears, therefore, that soleus fibers need more time to be depleted of ATP than psoas fibers and our new procedure includes a depletion step of 2 days.

Second, are the ATPases that we measure confined to the myosin heads? In muscle, the energy cost is determined mainly by ATP hydrolyzing enzymes: actomyosin ATPases, sarcoplasmic reticulum Ca^{2+}-ATPase and, to a lesser extent, sarcolemmal Na^+-K^+-ATPase. It has been estimated that 10–40% of the total energy used in muscle contraction is required for ion pumping (MacIntosh et al., 2000, and references cited therein). In view of their mode of preparation (the fibers were left in 50% glycerol for at least 2 days at –20°C, and the myofibrils were treated with Triton X-100) (Kurebayashi and Ogawa, 1991; Szentesi et al., 2001), it seemed unlikely that the myofibrils were contaminated with these membrane ATPases. This was confirmed by the lack of inhibition by CPA (specific inhibitor for Ca^{2+}-ATPase) and ouabain (specific inhibitor for Na^+-K^+-ATPase). We conclude that the ATPases we measured refer to the myosin heads only.

Third, is 100 μM Ca^{2+} enough to fully activate the soleus ATPase over the whole temperature range of the Arrhenius plots? Thus, a possible explanation for the high energy of activation of the k_F of soleus myofibrils is that the K_d for Ca^{2+} increases with a decrease in temperature, i.e., it could be that at 4°C, the myofibrils are not fully activated at 100 μM Ca^{2+}. This is unlikely, because the myofibrils were activated to equal extents with 50 and 100 μM Ca^{2+} at 4° as well as 25°C.

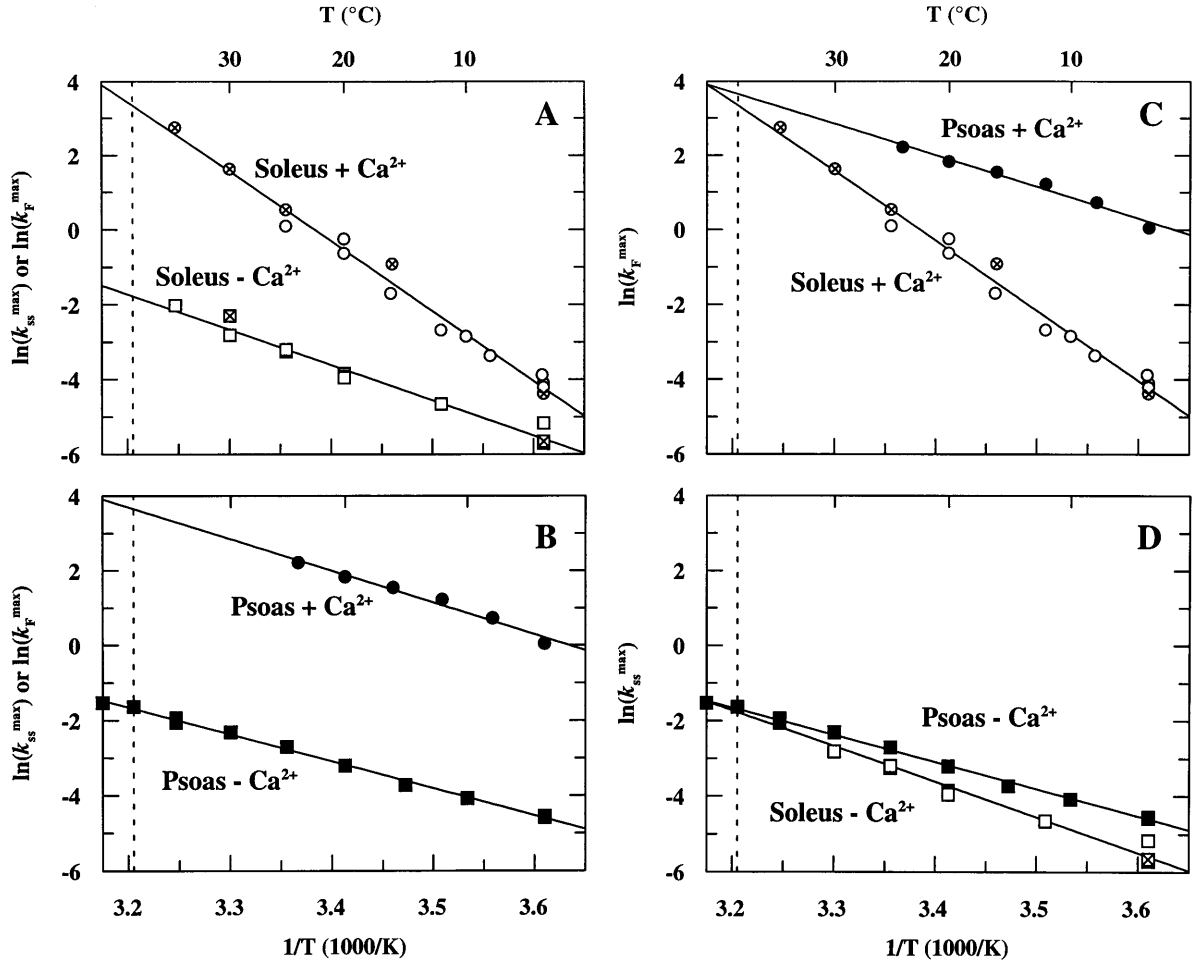

FIGURE 4 Temperature dependences of the ATPase steady-state rates of Ca^{2+}-activated (k_F^{max}, ○ and ●; $+Ca^{2+}$) or relaxed (k_{ss}^{max}, □ and ■; $-Ca^{2+}$) myofibrils from either soleus (*empty symbols*) or psoas (*filled symbols*). Crossed symbols indicate that these particular values are from extrapolation to infinite ATP concentration in K_m determination experiments, as illustrated in Fig. 3. For convenient comparisons, the scales are identical in all four panels. Comparisons of activated versus relaxed ATPases in soleus (*A*) and psoas (*B*) myofibrils. Comparisons of soleus versus psoas myofibrils for activated (*C*) or relaxed (*D*) ATPases. The vertical dashed lines correspond to the physiological temperature of the rabbit (39°C). The data for psoas myofibrils are from Lionne et al. (1999).

Finally, we must consider any effect of the build-up of ADP during contraction. With myofibrils, this is important because of the low ATP concentrations used. In skeletal muscle, the free ATP, ADP, and P_i concentrations are respectively ~4, 0.02, and 2 mM (Bagshaw, 1993). During intense and prolonged exercise, the ATP concentration decreases slightly, remaining above 2–3 mM, but the ADP and P_i concentrations increase by ~200 and 500%, respectively (Dawson et al., 1978; Hogan et al., 1999). These concentration changes appear to depend on the muscle type (e.g., Dahlstedt et al., 2000). In our experiments with myofibrils, the ATP concentration was typically 50 μM; nevertheless, for the duration of k_F (activated myofibrils) the accumulation of ADP was not sufficient to inhibit the ATPase (Table 2). With isometric skinned soleus fibers, added ADP (500 μM, ~3× myosin head concentration; ATP ~1.5 mM) inhibited the fiber ATPase, but the PCr/CK system had little effect (He et al., 1998). Here, with shortening myofibrils, neither added ADP (10 μM, 2× myosin head concentration; ATP = 50 μM) nor the PCr/CK system had any significant effect on k_F (Table 2).

Temperature dependences of the ATPases of soleus and psoas compared

k_F^{max} and k_{ss}^{max}

It is generally agreed that muscle contraction is linked with the product release steps (P_i, ADP) of the myosin head ATPase and that one or both of these limits the overall steady-state rate (e.g., Goldman, 1987). Therefore, any discussion of the myofibrillar ATPase must be in the context of this consensus.

We show here that the Q_{10} value for the ATPase of actively shortening soleus myofibrils (k_F^{max}) is remarkably high: 8.7 (E_a = 155 kJ mol^{-1}) compared to 2.7 (E_a = 71 kJ

TABLE 4 Energies of activation (E_a) for soleus and psoas myofibrillar ATPases and steady-state rates and activating factors extrapolated to 39 and 42°C

	$+Ca^{2+}$			$-Ca^{2+}$				
		k_F^{max} (s^{-1})			k_{ss}^{max} (s^{-1})		F_{activ}	
	E_a (kJ mol^{-1})	39°C	42°C	E_a (kJ mol^{-1})	39°C	42°C	39°C	42°C
Soleus	155 ± 6	28.3	50.1	78 ± 5	0.170	0.226	166	222
Psoas	71 ± 5	38.7	50.1	60 ± 3	0.182	0.226	213	222

mol^{-1}) for psoas myofibrils (12–30°C; Table 5). The Q_{10} values for the ATPase of the relaxed soleus and psoas myofibrils were similar and low (3.0 and 2.3, respectively).

Wang and Kawai (1997, 2001) studied the kinetics and thermodynamics of the cross-bridge cycle in skinned rabbit psoas and soleus fibers by sinusoidal analysis. They concluded that the elementary steps of the cycle are more temperature sensitive in the soleus than in the psoas. In particular, they showed that with soleus fibers, in the temperature range 20–37°C, the P_i release kinetics are highly temperature sensitive: $Q_{10} = 6.7$ ($E_a = 140$ kJ mol^{-1}), whereas with psoas fibers $Q_{10} = 3.3$ ($E_a = 89$ kJ mol^{-1}) (Wang and Kawai, 2001, and references cited therein). With the reservation that these works refer to the isometric condition, they are in overall agreement with effect of temperature on k_F (i.e., the ATPase of myofibrils shortening under zero external load, Table 5).

On the other hand, Wang and Kawai (1997) report that at 20°C, the P_i release kinetics with soleus fibers equals 5.7 s^{-1}, i.e., an order of magnitude greater than k_F (0.6 s^{-1}, Table 2). Further, they conclude that the overall ATPase of soleus fibers is limited by a slow isomerization of an AM·ADP state, i.e., by the overall ADP release kinetics (Wang and Kawai, 2001). Thus, it cannot be excluded that k_F is governed by the ADP release kinetics and that both the product release steps are highly temperature sensitive.

It is noteworthy that with S1 from MHC-II type fibers, the temperature sensitivity of the kinetics of the P_i release is low and that of the ADP release high (Trentham et al., 1976; Biosca et al., 1984) and there is a break in the Arrhenius plot of k_{cat}. It has been shown that S1 from MHC-I type fibers has a lower ATPase activity than S1 from MHC-II fibers (Weiss et al., 2001, and references cited therein), but a temperature study does not appear to have been carried out on this slow S1. Millar and Homsher (1992) proposed that with isometric soleus fibers the rate-limiting step is modulated by the temperature. In this event there could be a break in the Arrhenius plot of the fiber ATPase. But with unloaded soleus myofibrils, there was not a break (Fig. 4). A slow ADP release would be in accord with He et al. (1998), who suggest that the lower tension cost of slow fibers is explained by a slow ADP release.

Alternatively, it could be that with soleus myofibrils the rate-limiting step depends on the mechanical condition: isometric, ADP release kinetics; isotonic, P_i release kinetics. With rabbit psoas myofibrils, however, the P_i release kinetics are rate limiting, whether the mechanical state is relaxed, isometric (chemically cross-linked myofibrils), or shortening under zero load (Lionne et al., 1995, 2002).

A further difficulty in the interpretation of fiber and myofibrillar ATPases is that their temperature sensitivities may be species dependent. Thus, Stienen et al. (1996) report that with human muscle the Q_{10} of the ATPases are similar and low, whether from slow or fast fibers (Table 5).

K_m

To our knowledge, the K_m for ATP (with respect to the ATPase, k_F) with actively shortening soleus myofibrils has not been reported before. Shirakawa et al. (2000) record a K_m for a fluorescent analog of ATP, Cy3-EDA-ATP, of 1.9 μM with isometrically contracting soleus myofibrils at 20°C. Here, also at 20°C, for ATP, we estimate 5.3 μM (by interpolation, Fig. 3 *C*). These values are comparable, but, of course, the contraction conditions and nucleotides used were different.

The K_m for ATP is remarkably sensitive to the temperature (Fig. 3 *C*, Table 3). Thus, on going from 4 to 20°C, it increased 18×; with psoas myofibrils the increase was only twofold. It could be that the difference in the K_m for ATP between soleus and psoas myofibrils is related to the ATP regenerating system in slow and fast muscles: mainly oxidative processes in slow muscle (high mitochondrial enzyme activities) and mainly anaerobic processes in fast muscle (high glycolytic

TABLE 5 Comparison of Q_{10} values (12–30°C) for fast and slow skeletal muscle ATPases

			Q_{10}		
Experimental Animal	Model	Contraction condition	Fast	Slow	References
Human	Fibers	Isometric	2.6–2.8		Stienen et al. (1996)
Rabbit	Myofibrils	Relaxed	2.3	3.0	In text
		Unloaded shortening	2.7	8.7	In text

activities) (Åstrand and Rodahl, 1986). It is, however, difficult to come to any conclusion as to the physiological meaning of the difference because the K_m values are well below the concentration of ATP in muscle fibers (~4 mM).

From the dependence of V_{max} on the ATP concentration, Pate et al. (1992) determined a K_m for ATP of 14 μM at 10°C with soleus fibers. At the same temperature, we found a K_m for k_F of ~1 μM with soleus myofibrils (determined by interpolation, Fig. 3 *C*). This is similar to the situation with psoas fibers and myofibrils, namely that the K_m for V_{max} is greater than that for k_F (Cooke and Bialek, 1979; Lionne et al., 1996).

Ca^{2+}-activation factor (F_{activ})

Because of the different E_a of the ATPases of relaxed and Ca^{2+}-activated soleus myofibrils (k_{ss} and k_F^{max}, respectively), the Ca^{2+}-activating factor, $F_{activ} = k_F^{max}/k_{ss}$, is very sensitive to the temperature: 4 at 4°C and 166 at 39°C. With psoas myofibrils, F_{activ} was much less sensitive: 122 at 4°C and 213 at 39°C. This lower F_{activ} with soleus compared to psoas was also found by He et al. (1998) who reported values of 52 and 204, respectively, at 15°C using single permeabilized rabbit fibers.

Attempts to connect myofibrillar ATPases with muscle mechanics

At unphysiologically low temperatures, both the ATPase rates and mechanical properties are slower with slow than with fast muscle preparations. In particular, at 15°C and 20°C rabbit soleus myofibrils have a much slower rate of force generation than psoas myofibrils (Tesi et al., 2000). From our work, the ATPase rates (k_F) of the soleus and psoas myofibrils are, respectively, at 15°C, 0.19 s^{-1} and 4.0 s^{-1}, and at 20°C, 0.58 s^{-1} and 6.6 s^{-1}. Therefore, at these temperatures, mechanically slow myofibrils have chemically slow ATPases. With rat skinned fibers, Ranatunga (1998) showed that the Q_{10} for the optimal shortening velocity and power output were higher with slow than with fast muscle.

However, here we show that at physiological temperatures, the ATPase rates of myofibrils from a slow muscle (soleus) are very similar to those from a fast muscle (psoas). How can we reconcile this similarity with the different mechanical properties of the two types of muscle (e.g., Rome et al., 1988; Josephson, 1993; Bottinelli and Reggiani, 2000)? First, it cannot be excluded that even if the ATPase rates are similar, the relative proportions of the different intermediates that populate their reaction pathways are different, and that this difference explains certain of the different mechanical properties of the two types of muscle. Second, because the ATPases were obtained with myofibrils that shortened under zero external load, they may not reflect contraction under more physiological conditions. Finally, since myofibrils are skinned, an important part of the apparatus controlling the contractile process is absent. The properties of this apparatus appear to depend on the type of muscle. Thus, slow and fast muscles have different frequencies of action potential and, therefore, different Ca^{2+} concentrations in the cytosol (Åstrand and Rodahl, 1986).

To conclude, our work illustrates the importance of carrying out temperature dependence studies on biological systems. Very recently, Kawai (2003) reviewed the information that can be gained from studies on the effect of temperature on isometric tension.

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