

Role of Creatine Kinase in Force Development in Chemically Skinned Rat Cardiac Muscle

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ABSTRACT The influence of phosphocreatine in the presence or absence of MgATP and MgADP was studied in Triton X-100-treated thin papillary muscles and ventricular strips of the rat heart. The pCa/tension relationships, the pMgATP/tension relationships, and the tension responses to quick length changes were analyzed. The results show three major consequences of the reduction of the phosphocreatine concentration in the presence of millimolar concentrations of the MgATP. (a) The resting tension and the maximal Ca²⁺-activated tension were increased, and the pCa/tension relationship was shifted toward higher pCa values and its steepness was decreased; these effects were enhanced by the inclusion of MgADP. (b) The time constant of tension recoveries after quick stretches applied during maximal activation was increased, while the extent of these recoveries was decreased. (c) The study of pMgATP/tension relationships in low Ca concentrations showed that the decrease in phosphocreatine induced a shift toward higher MgATP values with no changes in maximal rigor tension or the slope coefficient; these effects were increased by the increase in MgADP and were independent of the preparation diameter. Thus, modifications of the apparent Ca sensitivity and resting and maximal tension when phosphocreatine is decreased seem to be due to an increasing participation of rigor-like or slowly cycling cross-bridges spending more time in the attached state. These results suggest that endogenous creatine kinase is able to ensure maximal efficiency of myosin ATPase by producing a local high MgATP/MgADP ratio.

INTRODUCTION

The role of phosphocreatine in the tension generation of heart muscle is important to study because of the high concentrations in which it is found in the cardiac cell. Its possible role in the tension decrease during hypoxia, however, has rarely been discussed. During hypoxia in heart muscle, a progressive decrease in twitch tension is followed by an increase in resting tension. These alterations in contractile activity are not directly related to variations in the concentration of Ca ions. Electrical measurements (Ventura-Clapier and Vassort, 1980a), as well as Ca measurements by aequorin bioluminescence (Allen and Orchard,

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1983), indicate that a decrease in the amount of Ca ions available for the activation of contraction is not responsible for the decrease in peak twitch tension. Moreover, the rise in resting tension is due to an increase in rigor tension (Ventura-Clapier and Vassort, 1981; Holubarsch, 1983) rather than to a contracture induced by an increase in Ca ions (Nayler et al., 1979). Decreases in ATP levels do not seem to be an explanation for the twitch tension decay, because small MgATP and MgADP changes generally occur later than the early tension decrease after hypoxia. Among other possible factors, a decrease in internal pH has often been proposed (Katz, 1977; Jacobus et al., 1982; Allen et al., 1985). However, no conclusive data are available to correlate the early tension decrease with a slight acidification (see Fig. 3 of Allen et al., 1985). The most significant changes occurring after hypoxia are a rapid drop in phosphocreatine accompanied by a comparable increase in inorganic phosphate, P_i (for example, see Dhalla et al., 1972; Allen et al., 1985). The P_i increase may in part contribute to the fall in twitch tension because it decreases Ca sensitivity and the maximal tension of skinned cardiac muscle (Herzig and Ruegg, 1977; Kentish, 1986), whereas the increase in creatine had no effect (Kentish, 1986). Indeed, two observations argue for a role of phosphocreatine. First, twitch tension decreases in parallel with the decrease in phosphocreatine (Ventura-Clapier and Vassort, 1980*a, b*); second, once the phosphocreatine concentration is low, rigor tension develops, even in the presence of millimolar concentrations of MgATP (Ventura-Clapier and Vassort, 1981).

Phosphocreatine is generally considered a "reservoir" of high-energy phosphates exerting only a buffering effect on the concentration of MgATP. However, this explanation is not sufficient, and an increasing number of experimental results suggest a more direct role of phosphocreatine and its involvement in the immediate regulation of tension. A more recent proposal, including the role of the phosphocreatine system in respiratory control and energy delivery, was depicted as the phosphocreatine transport system in muscle. The potential regulatory role of this system in cardiac muscle contraction is based on the assumed phenomenon of compartmentation of adenine nucleotides inside highly organized and differentiated muscle cells (for a recent review, see Walliman and Eppenberger, 1985; Bessman and Carpenter, 1985). In mitochondria, the creatine kinase isoenzyme is coupled to oxidative phosphorylation and participates in the compartmentation of adenine nucleotides in these structures (Jacobus and Lehninger, 1973; Saks et al., 1980; Jacobus et al., 1982; Moreadith and Jacobus, 1982; Gellerich and Saks, 1982). The MM-creatine kinase isoenzyme of muscle myofibrils plays an important role in supplying energy for contraction in heart muscle (Saks et al., 1976); this enzyme is functionally coupled to myofibrillar ATPase (Bessman et al., 1980; Walliman et al., 1984; Saks et al., 1984). Bessman et al. (1980) have also shown that cardiac myosin ATPase preferentially uses MgATP rephosphorylated by creatine kinase rather than MgATP in solution. The development of rigor tension is critically dependent upon the phosphocreatine and MgADP concentrations in EGTA-treated or Triton X-100-treated preparations, although there may be differences in the concentration range (McClellan et al., 1983; Veksler and Kapelko, 1984; Ventura-Clapier and Vassort, 1985; Miller and Smith, 1985). It has been observed in skeletal muscle that

a given low concentration of MgATP, maintained by the creatine phosphokinase system, is much more effective in inducing contraction than the same concentration of MgATP alone (Perry, 1954; Savabi et al., 1983, 1984). An MgATP-regenerating system (creatine phosphate plus creatine kinase) has been used to improve the skinned preparation of skeletal muscle (Godt, 1974) or heart muscle (Maughan et al., 1978). More recently, Ferenczi et al. (1984) reported the importance of the phosphocreatine plus creatine kinase-regenerating system in increasing the maximal velocity of shortening in skinned muscle fibers.

In this respect, it appeared interesting to study the consequences of a decrease in the phosphocreatine concentration on mechanical properties of heart muscle using chemically skinned preparations that simultaneously allow control of Ca, MgATP, and MgADP levels. Furthermore, it appeared critical to dissociate Ca-activated contraction from rigor tension when studying the effect of metabolic substrates on mechanical activity. Resting and maximal tension and the resulting tension responses after quick length changes were analyzed in thin, skinned rat papillary muscles bathed in solutions of various phosphocreatine, MgATP, and MgADP contents. We observed that a decrease in phosphocreatine produced an overall increase in tension for all pCa from 9 to 4.5; this increase was interpreted as being the sum of an increase in rigor tension and a decrease in active tension as shown by the responses to quick length changes. The decrease in active tension could participate in the decrease in twitch tension observed after hypoxia. The results also suggest that the phosphocreatine plus creatine kinase system is a potent system to maintain a high MgATP/MgADP ratio near the active site of myosin ATPase and that a reduction in phosphocreatine produces modifications in all the parameters of contraction, probably by slowing the cross-bridge cycle. A preliminary report of some of the results has appeared (Vassort and Ventura-Clapier, 1985).

METHODS

Cardiac ventricular strips or thin right papillary muscles from rats (<200 g body wt) were excised at room temperature in a low-Ca (0.65 mM) Krebs solution, pH 7.4. Muscles were tied and mounted horizontally between two stainless steel hooks. One hook was glued to an AME (Horten, Norway) transducer; the bandwidth of the transducer and hook was 2 kHz. The other hook was glued to the lever of a vibrator (GW1, Gearing Watson, Eastbourne, England). This permitted 80% length changes in <2 ms. Both were coupled to a digital storage oscilloscope (OS4020, Gould, Inc., Cleveland, OH) connected to a computer (HP-85, Hewlett-Packard Co., Palo Alto, CA). Tension was also monitored on a paper recorder (Gould, Inc.). The vibrator was driven by a stimulator (Tektronix, Inc., Beaverton, OR). The length changes were monitored through a second AME transducer connected with a light glass rod to the lever of the vibrator. The vibrator and the strain gauge were fixed on a micromanipulator. It was thus possible to immerse the muscle in small chambers containing 2.5 ml of solution. Eight chambers were arranged around a disk that could be moved under the muscle to change the solution as required. The disk was itself immersed in a temperature-controlled bath positioned on a magnetic stirrer. Each solution was vigorously stirred at high speed (>1,000 rpm) to facilitate diffusion of Ca, EGTA, and substrates into the muscle. In any case, the speed was chosen so that no further change was observed in tension when the speed of stirring was increased. The speed of stirring had a large effect on the rate of change of tension but had no effect at high speed on the level of tension attained.

After being pinned and gently stretched to a length below the L_{\max} level, all muscles were subjected to 30 min incubation in a modified Krebs solution containing 10 mM EGTA. The muscles were then transferred into a relaxing solution (solution A, Table I) containing 1% vol/vol Triton X-100 and incubated for 1–2 h. After skinning, the muscle was washed in solution A and then transferred to fully activating solution (solution B or F). The length and diameter were determined optically with a binocular microscope and a micrometer. The mean diameter was $167.7 \pm 9.4 \mu\text{m}$ ($n = 22$) (the diameter ranged from 0.1 to 0.25 mm, except for the muscles shown in Fig. 10). The sarcomere length was adjusted using laser diffraction to 2.0–2.1 μm (10-mW HeNe laser, Spectra Physics, Inc., Mountain View, CA).

Relaxing and activating solutions were calculated using the computer program of Fabiato and Fabiato (1979) and the binding constants of Fabiato (1981). Imidazole (30 mM) and acetic acid were used as a buffer to adjust solutions to pH 7.1 and acetate was preferred to chloride anions because the latter are inhibitors of the reverse reaction of creatine kinase (Watts, 1973). The total EGTA concentration was 10 mM; the total Na content was 30 mM. Ionic strength was adjusted to 0.16 M with K acetate. Free concentrations of Mg and Ca and MgATP concentrations are expressed as $\text{pX} = -\log_{10} X$. Free Mg was kept at $\text{pMg} 2.5$. All experiments were performed at 22°C. The compositions of the solutions used in this study are listed in Table I. Intermediate concentrations were obtained by mixing two solutions of extreme concentration. Solutions were prepared daily from frozen stock solutions. Stock solutions of K_2EGTA and $\text{Ca-K}_2\text{EGTA}$ were made according to Fabiato and Fabiato (1979); the purity of EGTA was taken into account and the titration of the solutions was made according to Bers (1982). There was a good correlation between our calculated concentrations and the measured Ca concentrations as determined using a Ca electrode and a pH meter between $\text{pCa} 7$ and 5. $\text{pCa}/\text{tension}$ relationships were always established from $\text{pCa} 9$ to $\text{pCa} 4.5$. Because of some decline in tension with time, maximal tension was obtained with the standard activating solution (solution B) before each test solution. Commercially available ADP was checked for contamination with ATP by fluorimetric assay of MgATP (Lowry and Passoneau, 1972); K-ADP was used because it contained no traces of ATP as tested by fluorimetric assay. A stock solution of 50 mM K-ADP was mixed with an equimolar concentration of Mg-acetate. P_1, P_5 -diadenosine pentaphosphate (250 μM) was always present with ADP to avoid ATP generation by myokinase activity; it was also present in the control solutions for these experiments. Substrates were purchased from Boehringer-Mannheim (Munich, FRG). Salts were obtained from Merck (Darmstadt, FRG) or Prolabo (Paris, France) and EGTA was obtained from Sigma Chemical Co. (St. Louis, MO). Phosphocreatine (Neoton, Schiapparelli Farmaceutica, Turin, Italy) was a kind gift of Prof. E. Strumia.

Quick length changes were performed when tension had reached a plateau after a change in solution. Two typical phases could be distinguished: F_1 , the increase (or decrease) in tension in phase with the length change characterizing the elastic phase, and F_2 , the maximal recovery of tension. Data were analyzed using a basic HP-85 program and normalized to the maximum active tension, F_0 , when phosphocreatine (12 mM) and ATP (3.16 mM) were present. The percentage of recovery ($r\%$) was defined as:

$$r\% = \frac{(F_2 - F_1)}{(F_1 - F_0)} \times 100.$$

In each solution, five to seven stretches or releases were obtained and the results were computed.

All values are given as means \pm SEM and the results were analyzed using Student's t test.

RESULTS

Effect of Different Levels of Phosphocreatine at Constant pCa

Resting tension in solution A (pCa 9, Table I) was $8.8 \pm 1.8 \text{ mN} \cdot \text{mm}^{-2}$ ($n = 21$). The standard activating solution (solution B) contained both MgATP (pMgATP 2.5) and phosphocreatine (12 mM). No solutions contained added creatine kinase because no modification of the pCa/tension relationship has been observed on freshly dissected muscles in the presence of added creatine kinase (0.5 mg/ml). When the fiber was incubated in activating solution (solution B), tension slowly rose until it reached a steady state. The average maximal Ca-activated tension was $76.2 \pm 12.9 \text{ mN} \cdot \text{mm}^{-2}$ ($n = 21$).

When the phosphocreatine concentration was decreased stepwise from 12 to 0 mM at constant pCa (pCa 4.5) and with pMgATP 2.5, tension increased (Fig. 1). After the change in phosphocreatine, tension rapidly increased and often

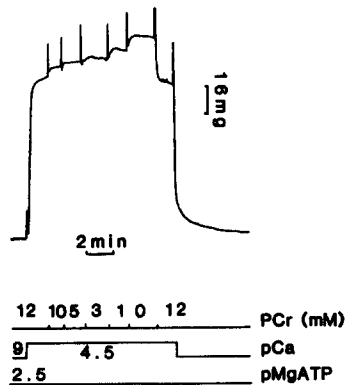


FIGURE 1. Continuous force recording obtained at pCa 4.5 and pMgATP 2.5 on Triton X-100-treated ventricular muscle of the rat in the presence of different phosphocreatine concentrations. Fiber diameter, $150 \mu\text{m}$; resting tension, 16 mg; maximal tension, 165 mg.

exhibited a slow decline. On return to the control solution (solution B), maximal tension was always decreased compared with its value at the beginning of the experiment. The mean increase in tension from 12 to 0 mM phosphocreatine was 34% (Table II).

Effect of Phosphocreatine and MgATP on pCa/Tension Relationships

Triton X-100-treated cardiac muscles were bathed in EGTA-buffered solutions of increasing Ca concentration obtained by mixing solutions A and B. Tension increased as the pCa was decreased over the range 6.5–5, Ca-activated tension being zero at pCa 9 and maximal at pCa 4.5. Data were fitted using the Hill equation: T (relative tension) = $[\text{Ca}]^{n_H}/K + [\text{Ca}]^{n_H}$. The Hill coefficient, n_H , and the pCa for half-maximal activation, $\text{pCa}_{50} = (-\log_{10} K)/n_H$, were calculated and the averaged values are listed in Table II. Fig. 2 shows the pCa/tension relation obtained for one typical fiber. The mean pCa_{50} and n_H coefficient were calculated from the values obtained for each pCa/relative tension relationship in each

experiment with solutions A and B and were, respectively, 5.731 ± 0.036 and 3.10 ± 0.14 ($n = 8$, Table II).

In the standard activating solution (solution B), the substrate for myosin ATPase arises from two different pathways: either directly from the diffusion of a physiological concentration of MgATP (3.16 mM) or through the rephosphorylation of MgADP by the endogenous creatine kinase present in Triton X-100-skinned cardiac preparations (Ventura-Clapier and Vassort, 1985; Ventura-Clapier et al., manuscript submitted for publication). To separate the two sources of MgATP, we studied the role of the diffusion of MgATP by adding a

TABLE II
*Characteristics of the pCa/Tension Relationship in the Rat
in Response to Changes in Phosphocreatine, MgATP, and MgADP*

Solutions	A-ADP and		C and D	C-ADP and	
	A and B	B-ADP		D-ADP	O and P
pMgATP	2.5	2.5	2.5	2.5	1.92
Phosphocreatine	12 mM	12 mM	0 mM	0 mM	0 mM
MgADP		10 mM		10 mM	
Resting force (%)			5.4	61	2.63
SEM			1	4.2	0.45
<i>n</i>			4	3	5
Maximal force (%)	100	132	134	144	118
SEM		3.2	9.4	7.9	0.6
<i>n</i>		3	4	3	5
pCa ₅₀	5.731	5.79	5.907	6.84	5.846
SEM	0.036	0.08	0.014	0.35	0.030
<i>n</i>	8	3	4	3	5
		NS	$P < 0.01$	$P < 0.01$	$P < 0.01$
<i>n</i> _H	310	3.033	2.14	0.92	2.59
SEM	0.14	0.033	0.36	0.09	0.18
<i>n</i>	8	3	4	3	5
		NS	$P < 0.01$	$P < 0.01$	$P < 0.01$

*n*_H and pCa₅₀ have been calculated as the sum (\pm SEM) of the values fitted for each experiment. The diameters of the preparations were between 0.10 and 0.22 mm. NS, not significant.

physiological concentration of MgATP (3.16 mM, pMgATP 2.5) after withdrawal of phosphocreatine. In the presence of MgATP (solutions C and D), the pCa/tension relation was modified by removal of phosphocreatine (Fig. 2A). Resting tension at pCa 9 was slightly increased by $5.4 \pm 1\%$ ($n = 4$). The maximal total tension was increased to $134 \pm 9.4\%$ ($n = 4$). When the pCa/tension relationship was normalized from 0 for pCa 9 to 100% for pCa 4.5 (Fig. 2B), the curve appeared shifted to the left, and showed an apparent increase in Ca sensitivity to a pCa₅₀ of 5.907 ± 0.014 ($n = 4$) (Table II). The slope coefficient of the curve was decreased to 2.14 ± 0.36 ($n = 4$), which suggests that the phosphocreatine supply may greatly influence the steepness of the pCa/tension relationship.

To investigate the additional influence of the total concentration of energy-rich phosphate bonds on active tension, the concentration of MgATP was raised to 12 mM (pMgATP 1.92; solutions O and P) in the absence of phosphocreatine; the pCa/tension relationship was compared with the one obtained in a solution containing 12 mM phosphocreatine and 250 μ M MgADP. Even in the presence of 12 mM MgATP, removal of phosphocreatine sometimes produced a slight increase in resting tension ($2.63 \pm 0.45\%$) and an increase in maximal force ($118.0 \pm 0.6\%$) ($n = 5$, Fig. 3 and Table II). The slope coefficient, n_H , was smaller (2.59 ± 0.18) and the pCa₅₀ was higher (5.846 ± 0.030) than in the standard activating solutions (solutions A and B), although the differences were

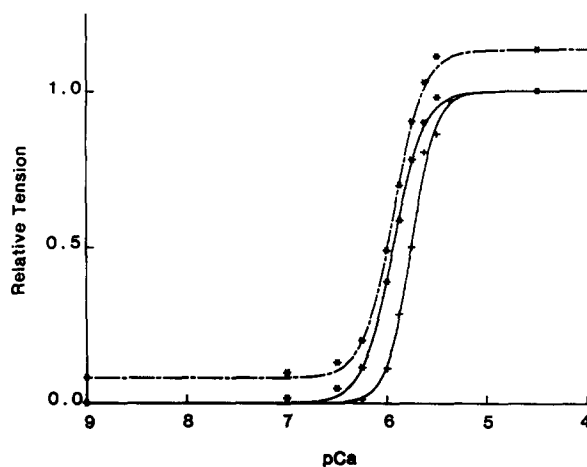


FIGURE 2. pCa/tension relationships obtained on the same fiber in the presence of 12 mM phosphocreatine and 3.16 mM MgATP (standard activating solutions [solutions A and B], crosses, continuous line), or after withdrawal of phosphocreatine (solutions C and D, stars, dashed line). In the latter case, resting tension and maximal tension are increased; this curve has been normalized to zero tension at pCa 9 and to 1.0 at pCa 4.5 (stars, continuous line). Sigmoid curves generated with the Hill equation (see text) yielded a pCa₅₀ of 5.75 and an n_H of 3.54 for the standard activating solution and a pCa₅₀ of 5.94 and an n_H of 2.87 after withdrawal of phosphocreatine. Fiber diameter, 120 μ m; resting tension, 8.5 mg; maximal tension, 68.5 mg.

less pronounced than when phosphocreatine was removed from the solutions with pMgATP 2.5 (Table II).

Effect of MgADP on pCa/Tension Relationships with and without Phosphocreatine

One hypothesis to explain the effect of phosphocreatine is that MgADP accumulates at the catalytic site of myosin ATPase and that creatine kinase may act by removing this MgADP. To investigate this possibility, a high concentration of MgADP (10 mM) was added (at constant pH, ionic strength, and pMg) in the presence and in the absence of phosphocreatine. In those experiments, P₁,P₅-diadenosine pentaphosphate (A_{p5}A) was added to inhibit myokinase in the control

and test solutions: no effect of $A_{p5}A$ itself was observed on the control pCa/tension relationship. In the presence of both MgATP and phosphocreatine (solutions A and B), 10 mM MgADP induced an increase in maximal tension to $132 \pm 3.2\%$. The Hill coefficient and pCa_{50} were not significantly altered (Table II).

The effects of 10 mM MgADP were much more pronounced when MgATP was the only substrate (solutions C-ADP and D-ADP). Resting tension at pCa 9 was increased to $61 \pm 4.2\%$ and maximal tension was increased to $144 \pm 7.9\%$. It can be seen after normalizing the curves that this was accompanied by a shift of the pCa/tension relationship from 5.731 ± 0.036 to 6.84 ± 0.35 ($n = 3$) toward higher pCa values and by a large decrease in n_H from 3.10 to 0.92. (Table II and Fig. 4).

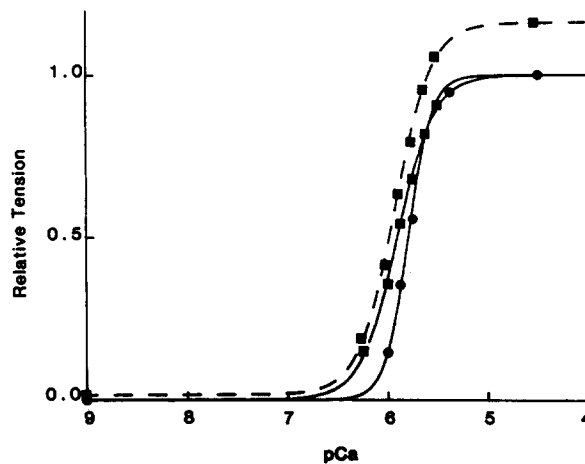


FIGURE 3. The pCa/tension relationship established in the presence of 12 mM phosphocreatine and 250 μ M MgADP (circles, continuous line) is compared with the tension/pCa relationship obtained from the same papillary muscle in the presence of 12 mM MgATP (pMgATP 1.92, squares, dashed line). The continuous line gives normalized tensions assuming zero tension at pCa 9. The curves have been drawn from the linearization of the Hill equation with pCa_{50} and n_H coefficients equal to 5.79 and 3.69, respectively, for the standard activating solutions (solutions A and B) and 5.90 and 2.26 for the solution containing 12 mM MgATP (solutions O and P). Fiber diameter, 100 μ m; resting tension, 7.2 mg; maximal tension, 32 mg.

Dependence of Rigor Tension upon MgATP, Phosphocreatine, and MgADP

For very low Ca concentrations, we often found that when phosphocreatine was omitted, there was a slight increase in resting force in the presence of 3.16 mM MgATP, the amplitude of which depended on the diameter of the preparations. We tested the hypothesis that this increase in force most probably reflected the increase in rigor tension as MgATP was decreased. We studied the influence of phosphocreatine, MgATP, and MgADP on pMgATP/tension relationships at a low Ca concentration (pCa 9).

A pMgATP/tension relationship was established by varying MgATP from 1 μ M to 12 mM (Fig. 5). The curves were fitted by an inverse Hill equation. In the absence of phosphocreatine, the pMgATP for half-maximal relaxation (pMgATP₅₀) was 3.71 and n_H was 2.03. Maximal rigor tension at pMgATP 6 was identical whether or not phosphocreatine was present. When phosphocreatine was present, the pMgATP/tension relationship was shifted toward higher pMgATP values (pMgATP₅₀ = 5.17; n_H = 2.63). Thus, the pMgATP₅₀ increased by 1.46 units when MgATP was provided by the rephosphorylation of MgADP through the creatine kinase present inside the muscle, as compared with the situation in which MgATP was added to the medium.

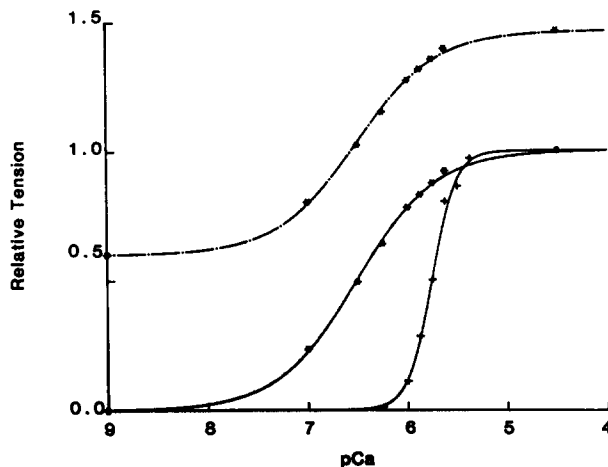


FIGURE 4. Effects of 10 mM MgADP on pCa/tension relationships. In the absence of phosphocreatine, the addition of 10 mM MgADP (solutions C-ADP and D-ADP) produced a much larger increase in resting and maximal tension (stars, dashed line) than removal of phosphocreatine alone (see Fig. 2). Normalized curve (stars, continuous line). Standard activating solutions (solutions A and B; crosses, continuous line). The pCa₅₀ and n_H values are shifted, respectively, from 5.75 and 3.54 to 6.50 and 1.09 in this experiment. Same fiber as in Fig. 2.

When 10 mM MgADP was added in the absence of phosphocreatine (Fig. 5), the pMgATP/tension relationship was shifted toward lower pMgATP values, which showed an increase in rigor tension for millimolar MgATP concentrations, but no increase in the maximum rigor tension was observed. The linearization of the pMgATP/tension relationship shows that pMgATP₅₀ was decreased to 2.87 with no significant change in steepness. Thus, when phosphocreatine was absent, the accumulation of the end product of the myosin ATPase reaction increased rigor tension by exerting an inhibitory competition with MgATP for relaxation.

We further analyzed the role of creatine kinase in regard to the possibility that its presence near the ATPase sites may allow rapid and efficient removal of the end product of the myosin ATPase reaction. We investigated the effects of increasing MgADP from 0 to 20 mM on rigor tension elicited on the same

muscle in three different conditions. The MgADP concentration was increased first in the absence of phosphocreatine but with MgATP present (pMgATP 2.5; solution C; Fig. 6A), then in the presence of 12 mM phosphocreatine (solution E; Fig. 6B), and finally in the absence of both MgATP and phosphocreatine (Fig. 6C).

When rigor tension was induced by the withdrawal of phosphocreatine in the presence of 3.16 mM MgATP (pMgATP 2.5, solution C), the increase in MgADP from 250 μ M to 1 mM had no effect on rigor tension; further increases in MgADP from 2 to 20 mM induced an increase in rigor tension toward its maximum. The addition of phosphocreatine in the presence of 20 mM MgADP

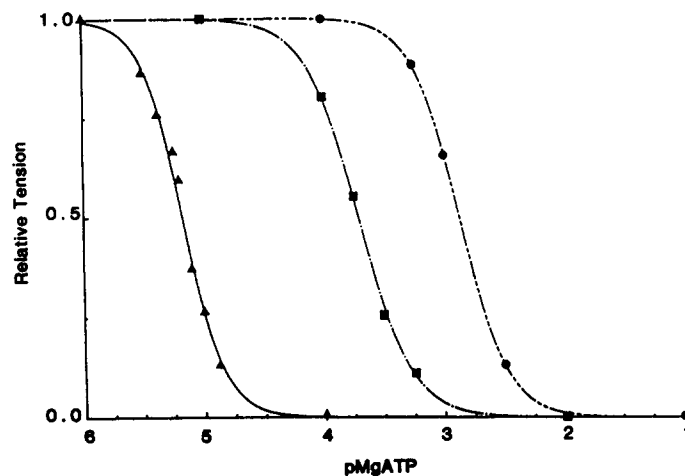


FIGURE 5. Comparison of pMgATP/tension relationships established in the absence of phosphocreatine with (circles) or without (squares) 10 mM MgADP and in the presence of 12 mM phosphocreatine (triangles) and no MgADP. This comparison emphasizes the shifts of the pMgATP for 50% relaxation of rigor tension produced by the absence of phosphocreatine and by the addition of MgADP. Fiber diameter, 150 μ m; resting tension, 11.2 mg; maximal tension, 234 mg.

completely relaxed the muscle (Fig. 6A). In Fig. 6B, maximal rigor tension was induced in the presence of 12 mM phosphocreatine by a decrease of MgATP from 3.16 mM to 1 μ M in the absence of MgADP. When 250 μ M MgADP was added, the muscle relaxed quickly and completely (Ventura-Clapier and Vassort, 1985). The further addition of 500 μ M up to 20 mM MgADP did not induce any rise in rigor tension. 250 μ M MgADP was sufficient to activate endogenous creatine kinase in the presence of phosphocreatine and to fully relax the muscle. In Fig. 6C, rigor tension was induced by the absence of MgATP and phosphocreatine; MgADP, whatever its concentration, did not modify rigor tension.

Responses of Tension to Quick Length Changes When Phosphocreatine Is Decreased

To further characterize the mechanical properties when the phosphocreatine concentration was varied, it appeared necessary to dissociate actively cycling

cross-bridges from rigor-like cross-bridges. This was done by studying the tension recoveries obtained after a sudden change in length (Heinl et al., 1974).

Quick length changes were produced in the fully activating solution (solution F, pCa 4.5). The resulting tension transients were compared for different phosphocreatine concentrations. Fig. 7 illustrates tension transients after a stretch of 1.6% of the initial length in the absence of MgATP. These tracings have not been corrected for the passive tension. After a quick stretch, tension increased to reach the maximal force value, F_1 , and then spontaneously decreased. Tension

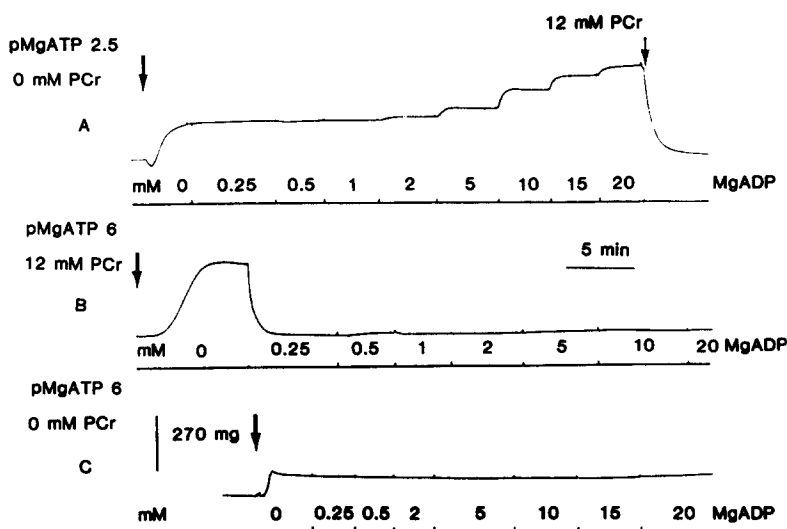


FIGURE 6. Tension recordings of a Triton X-100-treated papillary muscle in the absence of Ca (pCa 9), showing the dual role of MgADP in the relaxation of rigor tension. (A) Partial rigor tension is induced by the withdrawal of phosphocreatine in the presence of 3.16 mM MgATP (pMgATP 2.5). Increasing MgADP from 250 μ M to 20 mM induced an increase in rigor tension that was fully relaxed by 12 mM phosphocreatine. (B) In the presence of 12 mM phosphocreatine, rigor tension was induced by withdrawal of MgATP (10^{-6} M) when no MgADP was present. The addition of 250 μ M MgADP fully relaxed rigor tension by activation of endogenous creatine kinase. A further increase in MgADP up to 20 mM did not induce rigor tension. (C) In the absence of phosphocreatine and MgATP (pMgATP 6), MgADP had no effect on rigor tension. Fiber diameter, 200 μ m; resting tension, 83 mg; maximal tension, 678 mg.

recovery, $F_2 - F_1$, was larger when phosphocreatine was 12 mM and decreased with lower concentrations of phosphocreatine. Without phosphocreatine, the tracings no longer exhibited tension recoveries. This response is typical of the response of muscle in rigor. Thus, the tension recoveries show that when phosphocreatine is decreased, tension undergoes a gradual transition from active tension to rigor tension, with a decrease in the capacity of the muscle to develop active tension.

The F_1 and F_2 curves derived from these experiments are shown in Fig. 8 for two different phosphocreatine concentrations: 12 and 1 mM in the absence of

MgATP (solutions F and H). Passive tension responses have been obtained at pCa 9 by applying quick length changes. F_{1p} (F_1 for passive tension) and F_{2p} (F_2 for passive tension) have been plotted in Fig. 8 and have been subtracted from the F_1 and F_2 values obtained at pCa 4.5. Furthermore, tension responses have been normalized to the maximal tension response obtained in standard activating solution (solution B) so that we could compare the steepness of the F_1 curves. When phosphocreatine was decreased, this steepness increased, which suggests an increase in the stiffness of the muscle. With decreasing phosphocreatine

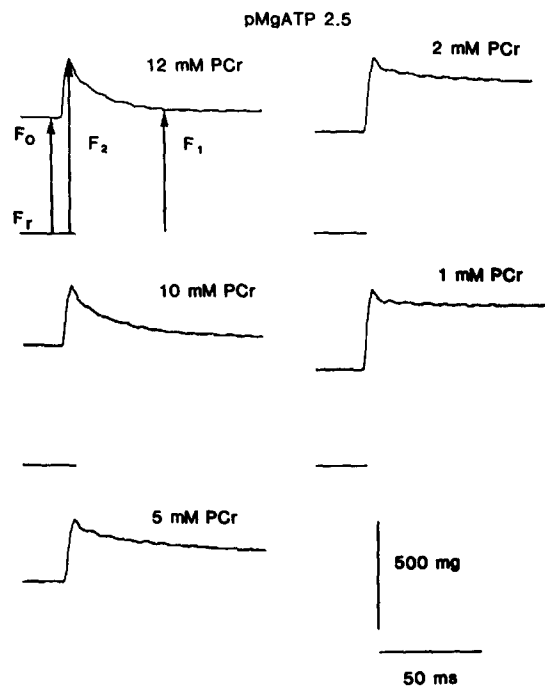


FIGURE 7. Original tension recordings of the responses of a fully activated ventricular strip to a quick stretch of 1.6% of the maximal length for decreasing concentrations of phosphocreatine (solutions B and D) in the presence of pMgATP 2.5. F_r , F_0 , F_1 , and F_2 are, respectively, the resting force, the maximal force, the force attained during the elastic phase, and the force attained after the spontaneous tension recovery. The amplitude of the spontaneous tension recovery ($F_2 - F_1$) decreased and slowed down when the phosphocreatine concentration was decreased. Fiber diameter, 230 μm ; resting force, 38 mg; maximal force, 563 mg.

concentrations, the difference between the F_1 and F_2 curves disappeared as a consequence of reduced tension recoveries.

The extent of recovery has been calculated as the ratio between recovery from F_1 to F_2 to the extent of the tension change $F_1 - F_0$ (see Methods). The extent of recovery as a function of length changes and for different phosphocreatine concentrations is shown in Fig. 9. In Fig. 9A, MgATP was present at 3.16 mM (solutions B and D); in Fig. 9B, low MgATP was present at 10^{-6} M (solutions F

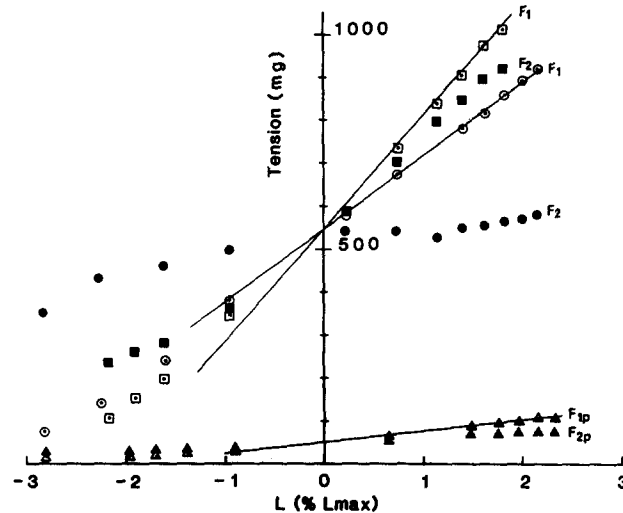


FIGURE 8. Forces plotted against the amplitude of stretches or releases, expressed as a percentage of maximal length. The open symbols are the elastic phase (F_1 curves); the filled symbols are the recovery phase (F_2 curves). F_1 and F_2 values for passive tension (triangles) were subtracted from values measured during activation. The circles indicate standard activating solutions; the squares indicate tensions in the absence of phosphocreatine. To show the change in the stiffness of the muscle, F_0 values, in the presence and in the absence of phosphocreatine, were normalized. Same fiber as in Fig. 7.

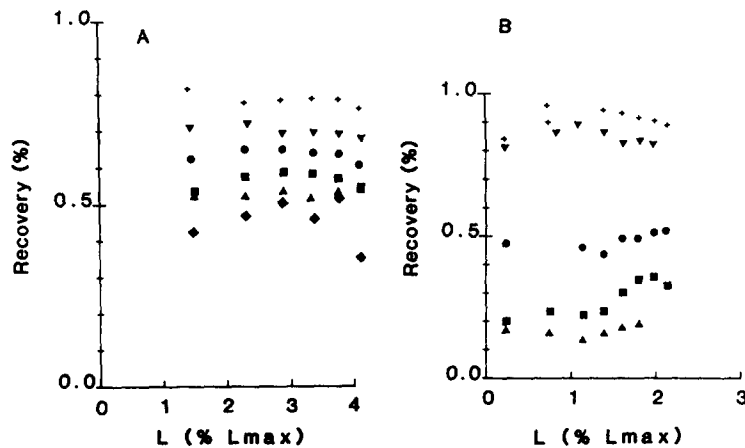


FIGURE 9. Effects of different phosphocreatine concentrations on the extent of recovery after quick length changes at $\text{pMgATP } 2.5$ (A) or $\text{pMgATP } 6$ (B). The extent of tension recovery decreases in both cases with the decrease in phosphocreatine but is decreased less at low MgATP concentrations. (A) Fiber diameter, $150 \mu\text{m}$; resting tension, 16 mg ; maximal tension, 165 mg . (B) Fiber diameter, $230 \mu\text{m}$; resting tension, 38 mg ; maximal tension, 563 mg .

and H). With 12 mM phosphocreatine and either pMgATP 2.5 (solution B) or pMgATP 6 and 250 μ M MgADP (solution F), the percentage of recovery was not significantly different. The recoveries were roughly constant with the increase in length and decreased with the decrease in phosphocreatine; the decrease was slightly less pronounced when 3.16 mM MgATP was present (Fig. 9B). These effects were partially reversible when the phosphocreatine concentration was restored. After going into rigor, the muscle never fully recovered the control level of recovery or the maximal tension, as if the fiber had suffered some irreversible damage.

Furthermore, a time constant of recovery was estimated as the time for an e-fold change in tension between F_1 and F_2 . This value has been calculated between two points taken at 63% recovery ($T_{63\%}$) and 90% ($T_{90\%}$) recovery using the formula $T = (T_{90\%} - T_{63\%})/\ln 3.7$. In this range, tension recoveries appeared mostly exponential and devoid of oscillations or stimulation artifacts. The time constant was increased from 12.23 ± 0.49 ms ($n = 16$) to 35.6 ± 2.4 ms ($n = 7$) for 1% stretches, when the phosphocreatine concentration was decreased from 12 to 0 mM. In the presence of both MgATP and phosphocreatine, 10 mM MgADP slowed the tension recovery slightly and the time constant of recovery was then 21.2 ± 1.95 ms ($n = 4$). Furthermore, after removal of phosphocreatine in the presence of high MgADP (10 mM), the time constant was no longer measurable because the recoveries were too small.

No evidence of tension recovery was obtained when quick length changes were applied at low MgATP and low Ca concentrations in the absence of phosphocreatine; the stiffness of the muscle was increased (results not shown), which suggests that this tension was due to rigor cross-bridges (in contrast to the findings of Miller and Smith, 1985).

DISCUSSION

In this study, we investigated the role of the endogenous creatine kinase bound in the myofilaments of skinned rat papillary and ventricular muscles, by decreasing the concentration of phosphocreatine in the presence of millimolar concentrations of MgATP. We showed that reducing the concentration of phosphocreatine produced an increase in the baseline tension and the maximal Ca^{2+} -activated tension and a shift of the pCa/maximal tension relationship toward higher pCa values, together with a decrease in the slope of the pCa/tension curve. These results are similar to those of Best et al. (1977), who studied the effects of decreasing the MgATP concentration in the presence of an ATP-regenerating system (phosphocreatine plus creatine kinase) in mechanically disrupted mammalian cardiac cells, and to the results of Brandt et al. (1982) on skinned psoas muscle fibers. In the latter case, however, no increase in maximal tension was observed. A similar shift in the pCa/tension relationship when pMgATP was decreased was also observed by Fabiato and Fabiato (1975) in mechanically skinned cardiac cells. In addition, responses to quick length changes show that, as phosphocreatine is lowered, the extent of tension recovery decreases and the time constant of tension recovery increases. It has been shown that tension recoveries are present during Ca-activated tension (Huxley and Simmons,

1971) and absent during rigor tension (Heinl et al., 1974). This suggests that fewer cross-bridges are generating active force and that their cycling is slowed down. All the effects are more pronounced with increases in MgADP. Consequently, when phosphocreatine is lowered, an increasing part of the maximal tension might be rigor tension caused by noncycling cross-bridges and a diminishing part may be due to active tension generated by slowly cycling cross-bridges. These results may be relevant to the decrease in force that occurs during hypoxia, because as phosphocreatine is decreasing, for the same internal Ca transient as in normal conditions (Allen and Orchard, 1983), fewer cross-bridges will be able to generate active force; the twitch tension will be decreasing while baseline tension increases because of rigor bridges. The close correlation between the decrease in twitch tension decrease in phosphocreatine and the increase in P_i reported in living heart muscle during hypoxia, ischemia, hypodynamia, or FDNB inhibition (Gercken and Schlette, 1968; Gudbjarnason et al., 1970; Dhalla et al., 1972; Saks et al., 1976; Ventura-Clapier and Vassort, 1980*a, b*) may be attributed in part to an increase in P_i (Kentish, 1986) and in part to a decrease in phosphocreatine.

Maximal Tension, Ca Sensitivity, and Slope Coefficient of the pCa/Tension Relationship

In heart muscle, maximal Ca-activated tension is increased by a decrease in MgATP in the presence of phosphocreatine (Best et al., 1977) or by a decrease in phosphocreatine in the presence of MgATP, whereas it is not increased in psoas muscle (Brandt et al., 1982) when the MgATP concentration is decreased from 5 to 0.25 mM. As shown by the F_1 curves (Fig. 8), the stiffness of the fibers is increased when phosphocreatine is decreased. This suggests that the number of bridges attached at any particular moment is higher than in active tension. Furthermore, the increase in the time constant of tension recovery suggests that the cross-bridge cycle is slowed down. Thus, the increase in maximal tension may be explained by an increased number of cross-bridges attached at any particular moment in the force-generating state when MgATP or phosphocreatine is decreased in heart muscle.

The pCa/tension relationships are thought to reflect Ca^{2+} binding to activating sites on troponin C. In our experiments, the Ca concentration required to obtain half-maximal tension was 1.86 μM in control solution, a value in good agreement with that reported for the low-affinity ($2 \times 10^{-6} M^{-1}$) site specific for Ca ions on troponin C (Holroyde et al., 1980). However, when MgATP and phosphocreatine are decreased, the increase in the apparent Ca sensitivity cannot easily be explained by a change in the affinity of troponin C for Ca ions. Two possible explanations have been discussed. Bremel and Weber (1972) showed that the affinity of troponin C for Ca increases with cross-bridge attachment. Brandt et al. (1982) suggested that the time cross-bridges spend in the attached and refractory states contributes to shifts in the pCa/tension relationships and must also be considered. With their model, they showed that the ratio of the dissociation rate constant for the Ca-troponin C complex to the rate constant for cross-bridge movement through the attached states affects the predicted midpoint of the pCa/tension relation. Because of the close association between myosin ATP-

ase and myofibrillar bound creatine kinase (see below), the decrease in phosphocreatine increases the number of rigor bridges and also increases the lifetime of the tension state by slowing the cross-bridge cycle. The pCa/tension relation thus must shift toward a higher pCa for the same reason that maximal Ca-activated tension increases.

Hill coefficients have no simple relation to the number of Ca^{2+} -binding sites and cannot be described simply by the binding properties of cardiac troponin C. As discussed by Brandt et al. (1980), they are merely used as a practical way to describe and compare the pCa/tension relationship. The coefficients we obtained in standard activating solutions were higher than the values reported for guinea pig trabeculae (Kentish, 1984) but lower than the ones reported for skeletal muscle (Brandt et al., 1982). These high Hill coefficients do not seem to depend on EGTA purity (see Methods). The Hill coefficients were decreased when phosphocreatine and MgATP were reduced. Although this was always associated with a higher apparent Ca sensitivity of the myofilaments in our experiments, the two observations could not be directly related. An increase in the apparent Ca sensitivity results in a shift of the pCa/tension relations toward pCa values where the two high-affinity ($2 \times 10^7 \text{ M}^{-1}$) binding sites for Ca on cardiac troponin C might also be participating; this would lead to an increase rather than to a decrease in the apparent Hill coefficient. Brandt et al. (1980) have proposed that the internal strain produced by the increased number of cross-bridges when Ca^{2+} is raised will slow down the cross-bridges with a progressive increase in tension, thus increasing the steepness of the pCa/tension relationship. We can infer that when cross-bridges are slowed down by substrate deficiency as the strain is already high (rigor tension), its influence on the steepness of the relationship will be decreased. These observations should draw attention to the importance of the regenerating system in the study of Ca regulation of contraction in heart muscle.

Creatine Kinase and Tension

The MM-isoenzyme of creatine kinase is present inside cardiac myofibrils (Scholte, 1973; Saks et al., 1976; Walliman et al., 1977). It is also present in Triton X-100-treated papillary muscles (2.2 U/mg protein; Saks et al., 1986; Ventura-Clapier et al., manuscript submitted for publication). The relaxation of rigor tension in skinned muscles depends more closely on the kinetics of creatine kinase and on the presence of its substrates, MgADP and phosphocreatine, than on MgATP (McClellan et al., 1983; Veksler and Kapelko, 1984; Ventura-Clapier and Vassort, 1985). The threshold of rigor tension was in the millimolar range. The most relaxed state was observed in the presence of 12 mM phosphocreatine and some MgADP (250 μM); relaxation was always maintained, in contrast to the observations of Miller and Smith (1985). It was also shown that a soluble ATP-regenerating system (pyruvate kinase plus phosphoenolpyruvate) produced only a slight shift of pMgATP/tension relation (Ventura-Clapier and Vassort, 1985), whereas a shift of 2 units was observed for the half-maximal relaxation of rigor tension in the presence of phosphocreatine and myofibrillar bound creatine kinase (McClellan et al., 1983; Veksler and Kapelko, 1984).

In the presence of Ca, phosphocreatine, with a low concentration of MgADP,

is able to maintain all the mechanical properties of skinned cardiac papillary muscles (Ventura-Clapier et al., manuscript submitted for publication), while a physiological concentration of MgATP alone cannot (Fig. 3). The endogenous creatine kinase proved to be very efficient in rephosphorylating the MgADP produced during activation of actomyosin ATPase by Ca. Increasing MgADP from 0 to 20 mM has a relaxing effect on rigor tension and only a small effect on active tension (Fig. 6) in the presence of phosphocreatine. However, MgADP increases rigor tension and induces marked changes in the pCa/tension relationship in the absence of phosphocreatine (Fig. 5), which indicates that the creatine kinase-phosphocreatine system is important not only in providing ATP but also in removing ADP from the active site of actomyosin ATPase.

Diffusion of hydrolyzable substances in skinned muscles leads to concentration gradients within the preparations (Meyer et al., 1984; Cooke and Pate, 1985). The ATP concentration is likely to decrease in the center of the preparations, while this profile could be strongly affected by adding phosphocreatine. This simple notion of facilitated diffusion, coupled with the observation that ADP itself leads to an enhancement of maximal force (e.g., Cooke and Pate, 1985) may explain most of the reported observations. However, we would expect that decreasing the diameter of the preparations would minimize the observed effects. We tentatively checked this hypothesis by looking at the variations in pMgATP_{50} , an estimate of the average effective ATP concentration in the whole preparation, as a function of preparation diameters in the presence or absence of phosphocreatine. Two major observations are illustrated in Fig. 10. Under both conditions, pMgATP_{50} increased slightly with decreasing diameter, as expected from restricted diffusion (Fig. 10A). However, the two sets of values always differed by at least 1.5 log units. Lower pMgATP_{50} values were also observed with high MgADP present, regardless of the diameter. When the difference was plotted against diameter (Fig. 10B), no correlation was found. The linear regression crosses the ordinate at pMgATP 1.64, which emphasizes that the difference in the pMgATP_{50} values appeared to be independent of the diameter of the preparation. This represents a 40-fold shift in the apparent K_M of the myosin ATPase; such an effect has also been reported by Krause and Jacobus (1986) in the ATPase activity of isolated myofibrils when phosphocreatine was added. If our observation is extrapolated to small diameters, it would suggest that the role of creatine kinase in supplying energy for cardiac contractions is not related solely to the diffusion distance in the preparation. Meyer et al. (1984) have proposed that the role of creatine kinase in muscle cells can be attributed to facilitated diffusion of adenine nucleotides between the cytoplasm and myofilaments. We would suggest that the role of creatine kinase bound in myofilaments, which has also been shown in isolated myofibrils (Saks et al., 1984), is important.

The half-maximal effect of MgATP obtained for relaxation of rigor tension was $\sim 500 \mu\text{M}$ in the absence of phosphocreatine and $10 \mu\text{M}$ in its presence. The latter value compares well with the highest activity of rat myofibrillar myosin ATPase ($14 \mu\text{M}$; Saks et al., 1984) and is in good agreement with that obtained on myofibrils or skinned fibers in skeletal muscle ($16 \mu\text{M}$; Glyn and Sleep, 1985). Interestingly, when MgADP is varied instead of MgATP, a similar value for half-

maximal relaxation is obtained ($13 \mu\text{M}$; Ventura-Clapier and Vassort, 1985); this value is lower than the reported K_M for MgADP of creatine kinase. The limitation in phosphocreatine decreases the rate of cycling of cross-bridges, and thus the ATPase activity, while fast cycling is observed when both MgATP and phosphocreatine are present. Goldman et al. (1984), using photochemical generation of

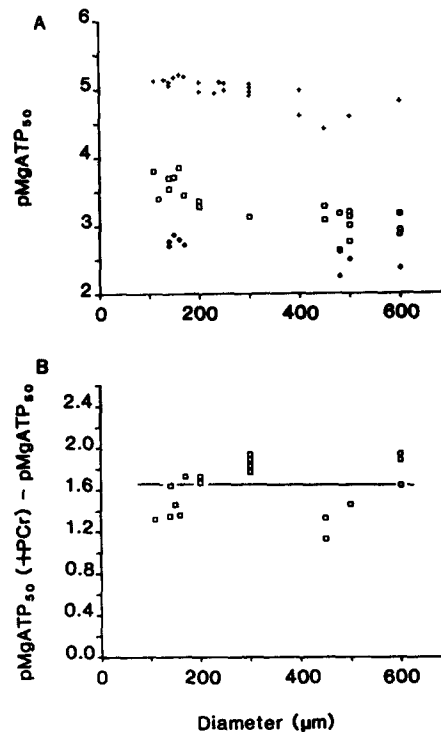


FIGURE 10. Influence of fiber diameter on the effectiveness of MgATP. (A) The concentrations of MgATP required for half-maximal relaxation of rigor tension (pMgATP_{50}) under three different experimental conditions are plotted as a function of the diameter of the fibers. (Crosses) pMgATP_{50} obtained in the presence of 12 mM phosphocreatine; (squares) pMgATP_{50} in the absence of phosphocreatine and MgADP; (diamonds) pMgATP_{50} in the absence of phosphocreatine but in the presence of 10 mM MgADP. (B) The differences in pMgATP_{50} in the presence and absence of 12 mM phosphocreatine are plotted against the fiber diameter. The straight line is the linear regression line. Slope, 0.000015; ordinate, 1.64; correlation coefficient, 0.0114.

MgATP, showed that ATP binding and cross-bridge detachment from the nucleotide-free intermediate of the cross-bridge are rapid compared with the cross-bridge cycling rate. Furthermore, Siemankowski and White (1984) have proposed that the rate constant of ADP dissociation from the acto-S1-ADP complex was slow enough to be the molecular step that limits the unloaded shortening velocity in cardiac muscle. Decreases in the time constant of recovery

both when only MgATP is present and when MgADP is added in excess are consistent with the proposal; the presence of a high amount of MgADP will slow down the detachment of MgADP, while the activity of creatine kinase will prevent this effect.

The role of creatine kinase bound to the myoflaments is to facilitate the detachment of MgADP by lowering its concentration around the actomyosin and by increasing the local MgATP concentration by rapid rephosphorylation. One other important effect may be also the pH-buffering capacity of the creatine kinase reaction, which will absorb H ions produced by the hydrolysis of ATP to rephosphorylate MgADP. This will prevent a local acidification, which will also decrease myosin ATPase activity. The results of the association of these two enzymes, all of which will permit a high myosin ATPase efficiency, will be a higher MgATP, a low MgADP, and constant pH.

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