THE DEPENDENCE OF ISOMETRIC TENSION, ISOMETRIC ATPase ACTIVITY, AND SHORTENING VELOCITY OF LIMULUS MUSCLE ON THE MgATP CONCENTRATION

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ABSTRACT The dependence of the isometric tension, the velocity of unloaded shortening, and the steady-state rate of MgATP hydrolysis on the MgATP concentration (range 0.01–5 mM MgATP) was studied in Ca-activated skinned Limulus muscle fibers. With increasing MgATP concentration the isometric tension increased to a peak at ~0.1 mM, and slightly decreased in the range up to 5 mM MgATP. The velocity of unloaded shortening depended on the MgATP concentration roughly according to the Michaelis-Menten law of saturation kinetics with a Michaelis-Menten constant K_v = 95 μM and a maximum shortening velocity of 0.07 muscle lengths s^{-1}; the detachment rate of the cross-bridges during unloaded shortening was 24 s^{-1}. The rate of MgATP splitting also depended hyperbolically on the MgATP concentration with a Michaelis-Menten constant K_a = 129 μM and a maximum turnover frequency of 0.5–1 s^{-1}. The results are discussed in terms of a cross-bridge model based on a biochemical scheme of ATP hydrolysis by actin and myosin in solution.

INTRODUCTION

Biochemical studies of the kinetics of the actin-activated ATP hydrolysis by myosin have revealed a detailed reaction scheme of the particular steps of the ATPase cycle. The intermediates and the transition rates of this cycle were identified and determined in solutions of purified myosin and actin from various muscles (rabbit psoas; for review cf. Taylor, 1979; Eisenberg and Greene, 1980; frog semitendinosus: Ferenczi et al., 1978*a*,*b*; slow and fast chicken myosins: Marston and Taylor, 1980; bovine cardiac muscle: Siemankowski and White, 1984).

Mechanochemical experiments on Ca-activated skinned muscle fibers were carried out to investigate the relationship between the concentration of MgATP and isometric tension, rate of MgATP hydrolysis, shortening velocity, and transient tension responses after quick changes of muscle length (rabbit psoas: Kawai, 1978, 1982; Cooke and Bialek, 1979; Goldman et al., 1982; frog semitendinosus: Ferenczi et al., 1982, 1984; Lethocerus dorsal longitudinal muscle: Wilson and White, 1983; Kuhn et al., 1985). The interpretation of the results of these experiments in terms of a kinetical cross-bridge model was attempted, but it is difficult to link the steps of the cross-bridge cycle within an intact filament lattice with the pathway of MgATP hydrolysis in solution since in fibers the kinetics of the cross-bridge cycle are strongly affected by mechanical constraints that do not exist in actin–myosin systems in solution. A probable way of overcoming these difficulties has been found by recent in vitro studies that suggest that the cross-bridges alternate between conformations that differ in their structure and in their affinity to actin when they bind to the actin (strong-binding states and weak-binding states of myosin or myosin–nucleotide with actin; cf. Eisenberg and Hill, 1985). It is thought that the mechanical constraints have an effect mainly on the strong-binding states of the cross-bridge cycle. In this study, we take a greatly simplified version of this biochemical scheme as the basis for interpreting our mechanical experiments on muscle fibers with respect to the mutual relationship between mechanical and chemical events during muscle contraction.

The well-ordered structure of the myosin filaments of Limulus muscle fibers (Wray et al., 1974) was seen to be a promising object for the studies of their cross-bridge kinetics. We describe experiments done on chemically skinned Limulus telson levator muscle in which we tested the effect of various MgATP concentrations on steady isometric tension and isometric ATPase activity as well as on shortening velocity. We compare the results with those obtained from other muscle species and from myosin and actin in solution.

METHODS

Solutions

The standard activating solution (and the test solutions derived from it) used to perform the measurements of isometric tension and shortening velocity was composed of the following constituents (mM): H_2Na_2ATP, 5 (Boehringer Mannheim GmbH, Mannheim, FRG); monosodium phosphoenylpyruvate (PEP), 15 (Boehringer Mannheim GmbH); $MgCl_2$, 11.3 (E. Merck, Darmstadt, FRG); $CaCl_2$, (E. Merck); EGTA, 4 (Serva Feinbiochemica GmbH & Co., Heidelberg, FRG); 1,6-diaminohexane-

N, N, N', N'-tetraacetic acid (HDTA), 10 (Serva Feinbiochemica GmbH & Co.); imidazole, 40 (Boehringer Mannheim GmbH); sodium azide (NaN_3), 10 (puriss.; Serva Feinbiochemica GmbH & Co.); and pyruvate kinase (PK), 1 mg/ml (Boehringer Mannheim GmbH) (activity 200 U/ml; the stock solution was dissolved in 50% [vol/vol] glycerol). The rigor solution was composed of the same constituents, except that H_2Na_2ATP was replaced by HDTA. The activating test solutions containing MgATP concentrations ranging from 0.01 to 5 mM MgATP were obtained by mixing the appropriate volumes of the standard activating solution and the rigor solution. In the relaxing solutions, $CaCl_2$ was replaced by KCl. In all test solutions the concentration of the energy-rich phosphates, MgATP + PEP = 20 mM, was maintained at a constant level; the ionic strength was adjusted to 150–155 mM by adding KCl to the solution; the pH was kept constant at 6.7 by adding KOH or HCl. The concentration of free Ca^{++} was calculated using Schwarzenbach's affinity constants of H^+ and Ca^{++} to EGTA at 22°C and 0.1 M ionic strength (cf. Anderegg, 1964). The calculations gave a pCa of 4.9 for the activating solutions, and pCa > 8 for the relaxing solutions ($pCa = -\log_{10}[Ca^{++}]$). The pMg value was adjusted to 2.8.

For determination of the rate of MgATP hydrolysis the following constituents were added to the standard solution (mM): nicotine amide adenine dinucleotide (NADH), 0.1–0.2 (grade I; Boehringer Mannheim GmbH); lactate dehydrogenase (LDH), 0.18 mg/ml (Boehringer Mannheim GmbH) (activity 100 U/ml); and the adenylate kinase inhibitor P^1, P^5-di(adenosine-5'-)pentaphosphate (AP_5A), 0.1 (Boehringer Mannheim GmbH).

Mechanical Set-up

For the measurement of the isometric tension and of the velocity of unloaded shortening, one end of the fiber bundle was attached with cellulose acetate glue to a glass rod connected to a length-step generator; the other end was wound around the force-transducer and glued to a fixed platinum needle.

Length changes were carried out with a Ling Dynamics 101 vibrator fed by a feed-back controlled power amplifier. Releases of 2–250 μm (corresponding to 0.05–7% of the initial fibers length L_i) were completed within 0.8 ms and lasted 3 s. To prevent mechanical damage to the fiber, the restretching procedure lasted 70 ms. The force-transducer (type 801; AME, Norway) was modified to have a resonance frequency of 10 kHz with the fibers attached. For a detailed description of the set-up see Güth et al. (1979, 1981).

The fiber bundles were incubated in a trough containing 0.5 ml of bathing solution. The solution was stirred by a magnetic stirrer. The temperature was maintained constant at 13° ± 0.5°C.

The long-term course of tension was recorded on a pen-recorded. The transient signals of the length change and the tension response to the release were displayed and sampled on a two-channel storage oscilloscope (Explorer 206; Nicolet Instrument Corp., Madison, WI).

Spectrophotometrical Measurement Device for the MgATP Activity

The steady-state rate of MgATP hydrolysis in isometrically contracting muscle fibers was determined spectrophotometrically using two linked enzyme systems (cf. Czok and Lambrecht, 1970). The phosphoenylpyruvate and the amount of ADP that originated from the splitting of ATP during the muscle contraction were rephosphorylated to ATP and pyruvate via pyruvate kinase. The pyruvate was linked to a second enzymatic system, which catalyzed the conversion of pyruvate and NADH to NAD and lactate using lactate dehydrogenase. The light extinction of the test solution (which is proportional to the NADH concentration) was measured with a spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) set at a wavelength of 340 nm.

Inside the assay chamber of the spectrophotometer, a rack was installed combining the holding support for the fibers and the photometer cuvette for the bathing solution. The holding support consisted of a glass rod and a force-transducer (type 801; AME, Norway) to which the fiber bundles were attached with cellulose acetate glue. The cuvette was connected with a closed tube system allowing the test solutions to be driven continuously through the cuvette by a peristaltic pump. The circulation of the bathing fluid minimalized the concentration gradients surrounding the fiber bundle, and a quick and easy exchange of the test solutions was possible (volume of the cuvette 200 μl, path length of light inside the cuvette 10 mm; volume of the total circulation system 250 μl; flow of solution 5 $\mu l\ s^{-1}$). The decrease of the extinction corresponding to the NADH disappearance during contraction and the concomitant time course of the isometric tension were monitored on a two-channel pen-recorder.

Before each series of ATPase measurements the signal of the NADH light extinction was calibrated to determine the extinction coefficient of the NADH assay without a muscle specimen inserted into the photometer cuvette. For this purpose, the rigor contraction solution, mixed with 0.1 mM NADH, was titrated consecutively with solutions of known ADP concentration yielding a mean extinction coefficient of $(6.42 \pm 0.04) \cdot 10^2$ $M^{-1}mm^{-1}$. Then the test solutions were freshly mixed with the enzymatic assay systems. They had to be examined spectrophotometrically for their ADP content, which could contaminate the ATP stock solution, to avoid a depletion of NADH immediately after the addition of the enzymes. With this control experiment, the final NADH concentration of the test solutions was sufficiently adjusted to saturate the rephosphorylation of ADP produced during contraction.

As soon as the fibers were glued to the holding support and inserted into the photometer cuvette, they were washed with rigor solution for 5 min. Then the enzymatic reactions were initiated by replacing the rigor solution with the MgATP-containing test solution. After a delay of 1–2 min the isometric tension reached a steady level, and the spectrophotometer showed a linear decrease of extinction.

The decline of light extinction by NADH per unit time was read from the pen-recorder plots and normalized to a single fiber 10-mm long. Using Beer's law, the normalized value was divided by the path length of the light inside the cuvette and the NADH extinction coefficient to give the change in the NADH concentration. This value was multiplied by the volume of the test solution to calculate the molar amount of ATP split during the isometric contraction.

Fiber Preparations

Thin strips (1–2-mm diam) were dissected from the medial telson levator muscle of the horseshoe crab Limulus, *Tachypleus polyphemus*, and chemically skinned by a 50% (vol/vol) glycerol-water mixture containing (mM) KCl, 40; $MgCl_2$, 8; EGTA, 2; H_2KPO_4, 5; and HK_2PO_4, 5 at pH 7. After the extraction procedure the fiber bundles were stored at 4°C (cf. Jewell and Rüegg, 1966).

All experiments were carried out on bundles consisting of three to five fibers. Using a dissecting microscope (50×), the diameter of a fiber bundle was estimated at three to seven sites along the length of the preparation and, assuming a circular cross-section, a mean cross-sectional area of the bundle was calculated. The cross-sectional area of the bundles varied between 0.01 and 0.08 mm^2.

The sarcomere length of the relaxed fibers was adjusted to 7.5–9.5 μm by means of the diffraction pattern obtained with a HeNe laser when the fiber bundle was mounted on its holding support. The initial length L_i between the supporting rod and the force-transducer was 3–6 mm. Care was taken to assure that this part of the bundle was clearly out of the zone of the fixing glue.

Evaluation of the Data

The experimental data of shortening velocity and of MgATPase activity fitted the Michaelis-Menten relation given by

$$y = A + B \cdot x/(K_M + x), \quad (1)$$

with y denoting the rate constants and x the MgATP concentration. By linear regression analysis starting with an arbitrary estimate of the

constant K_M, the coefficients A, B, and the variance were calculated. K_M was estimated from the minimal variance. This best fit was linearized with respect to K_M, and the standard errors (SE) of A, B, and K_M were calculated.

To describe the dependence of the isometric tension on the MgATP concentration, the linear combination of two hyperbolas,

$$F = F_0 + F_1 \cdot x/(K_{M1} + x) - F_2 \cdot x/(K_{M2} + x), \quad (2)$$

provided a good fit to the data found by experiment.

RESULTS

Isometric Tension

The dependence of the isometric tension on the MgATP concentration was measured over the concentration range 0.01–5 mM. Fig. 1 shows the tension responses to consecutive contraction–relaxation sequences at various MgATP concentrations recorded in a typical experiment. At first, the glycerinated fiber bundle was bathed in a relaxation solution (pCa > 8) containing 5 mM MgATP (in Fig. 1 *A*). When the relaxation solution was replaced by the contraction solution (pCa = 4.9, 5 mM MgATP) the fiber contracted reaching the tension plateau within 1 min. Such Ca-induced isometric contraction–relaxation sequences were repeated at various MgATP concentrations (Fig. 1, *B–E*).

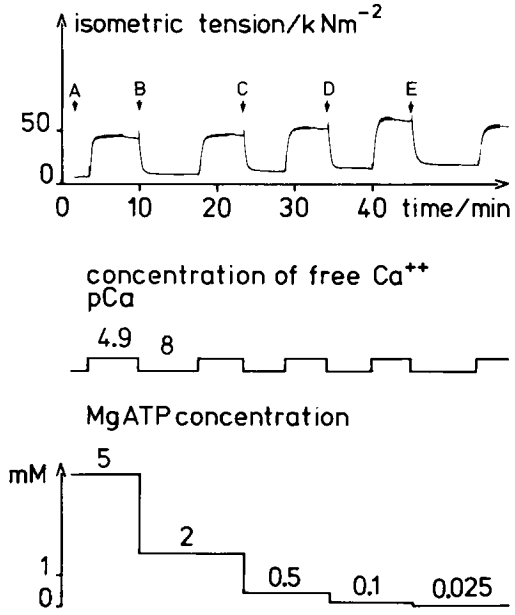


FIGURE 1 Dependence of the isometric tension on various MgATP concentrations in consecutive contraction–relaxation sequences (*upper trace*). Relaxation solutions: pCa > 8, contraction solutions: pCa = 4.9 (*middle trace*); MgATP concentrations as indicated (*lower trace*), the change of the MgATP concentration is marked by *A–E*. Steady-state levels of the isometric tension: 45.5, 46.3, 50.8, 57.4, and 49.7 kN m^{-2} at 5, 2, 0.5, 0.1, and 0.025 mM MgATP, respectively. Note the high stability of the tension plateaus achieved by stirring the bathing solution. Artifacts seen on the tension record are caused by a change of the bathing solution. The broadening of the trace shows the dynamic stiffness of the bundle (10 Hz). Experimental conditions: Initial length, 4.5 mm; cross-sectional area, 0.038 mm^2; sarcomere length, 9.1 μm; pH 6.7; temperature, 13°C; pMg 2.8, 200 U PK/ml (1 mg/ml).

In Fig. 2, the relative isometric tension of the Ca-activated muscle fibers (pCa 4.9) is plotted versus the MgATP concentration; the plateau value at 0.5 mM MgATP, 63 ± 6 kN m^{-2} (averaged over 11 experiments), was taken as a point of reference. The relationship of the isometric tension to the MgATP concentration was biphasic exhibiting a maximum at ~0.1 mM MgATP. During the relaxed state the tension levels did not noticeably depend on the MgATP concentration, except that at the concentration of 0.01 mM MgATP the rest tension was slightly increased.

This dependence of the isometric tension on the MgATP concentration can be mathematically described by the superposition of two hyperbolas of the Michaelis-Menten type. The graph of Fig. 2 was obtained by fitting Eq. 2 to the tension data. At low MgATP concentration, the best fit was obtained by a hyperbola with the Michaelis-Menten constant K_{M1} = 7.5 μM and the saturation value F_1 = 154%. At high MgATP concentrations, the second hyperbola yielded the Michaelis-Menten constant K_{M2} = 125 μM and the saturation value F_2 = 35.2%. At zero MgATP concentration, the fitted tension curve intercepted the ordinate at F_0 = −37%. This is in contrast to the experimental finding that the tension in the rigor state reached ~30% of the tension at 5 mM MgATP; therefore, the calculated negative F_0 value obviously has no physiological meaning.

Velocity of Unloaded Shortening

The velocity of shortening at zero load (Hill, 1970; Julian, 1971; Edman, 1979) of Ca-activated muscle fibers was studied at various MgATP concentrations ranging from 0.025 to 5 mM. During the plateau phase of isometric

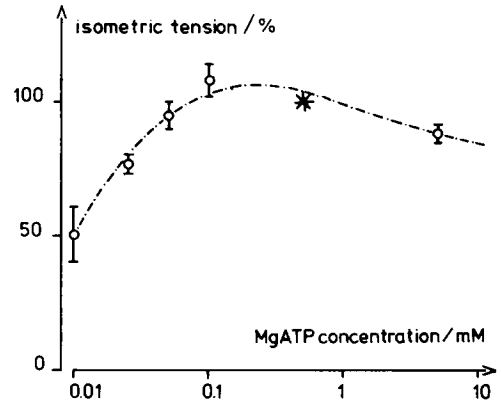


FIGURE 2 Effect of the MgATP concentration on the isometric tension. The ordinate is normalized to the tension value at 0.5 mM, 63 ± 6 kN m^{-2} (*); number of measurements 7–11, SE values as indicated. The graph is best fit of Eq. 2 (cf. Methods). Michaelis-Menten constants and saturation values calculated from the fit: K_{M1} = 7.5 μM and F_1 = 154%; K_{M2} = 125 μM and F_2 = 35.2%, F_0 = −37%. L_i, 3–4.5 mm; cross-sectional area, 0.01–0.08 mm^2; sarcomere length, 7.5–9.5 μm; pCa = 4.9, temperature, 19°C.

tension, the muscle fibers were quickly released, and tension was allowed to redevelop at the shorter length. Complete unloading was achieved in releases >1.5% of the initial fiber length. To determine the velocity of unloaded shortening, a series of releases with amplitudes in the range between 1.5% and 5% L_i were performed. Fig. 3 shows such an experiment carried out at a MgATP concentration of 0.5 mM.

Fig. 4 shows examples of tension transients measured during the redevelopment of tension after complete unloading at a MgATP concentration of 0.5 mM. Upon release, the tension dropped rapidly from the isometric tension level T_i to zero (T_0). The onset of tension marked the termination of the unloaded shortening. About 1 s after release, the curvature of the tension plots was fairly similar for the various amplitudes ΔL of the releasing step. An increase of ΔL resulted in a shift of the tension curves parallel to the ordinate to lower tension values without modifying their shape. This suggests that the kinetics governing the redevelopment of tension were not influenced by the extent of release.

The time Δt needed for the fibers to take up the slack was determined graphically from the plots of the tension transients, as can be read from Fig. 4. Δt was defined as the time interval between the moment when the zero tension level was reached and the time at which the unloaded shortening was terminated and tension started to redevelop. This point in time was estimated from the graphs. We took the intercept of the abscissa ($T_0 = 0$) with the tangent passing through the inflexion point of each curve at the tension re-uptake. Extrapolated in this way, the accuracy of Δt was limited by the methodological uncertainties inherent to graphical extrapolation; we estimated this inaccuracy to approximately ±5%. Recently, the problem of obtaining a more accurate estimate of Δt was addressed in detail by Julian et al. (1986).

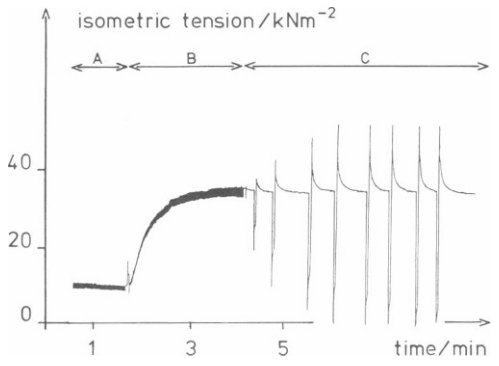


FIGURE 3 Tension record of slack tests for the determination of the velocity at zero load at a MgATP concentration of 0.5 mM. (*A*) Tension level in relaxation solution (pCA > 8). (*B*) Transfer of the fiber bundle to the contraction solution (pCa = 4.9) and tension rise, steady isometric tension 36.7 kN m^{-2}. (*C*) Consecutive tension transients following quick releases of various amplitudes ΔL (ΔL/percentage of L_i: 0.6, 1.2, 1.7, 2.2, 2.7, 3.3, 3.9, from the left). L_i, 4.3 mm; cross-sectional area, 0.049 mm^2; sarcomere length, 9.8; temperature, 13°C.

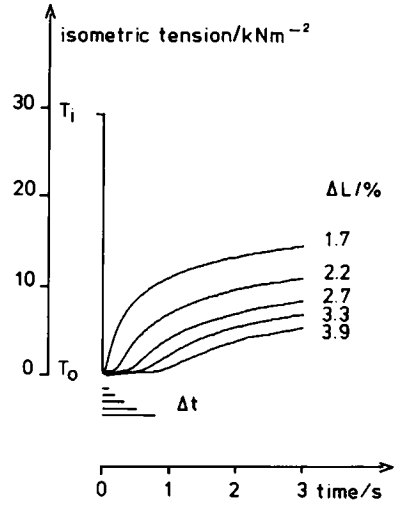


FIGURE 4 Superimposed oscilloscope records showing a series of tension transients after releases of various amplitudes. Seen is the fast tension decay from the maximum isometric tension T_i to zero tension T_0, the duration Δt of fiber slackening as a function of the length step amplitude ΔL (percentage of L_i, values as indicated), and the tension development after the re-uptake of tension. Same experiment as in Fig. 3.

In Fig. 5, the amplitude of release ΔL is plotted against the duration of unloaded shortening Δt for a series of various MgATP concentrations tested. The dotted lines are hyperbolas fitted to the data points. The higher the MgATP concentration, the steeper are the $\Delta L - \Delta t$ curves. With releases <3.5% L_i, Δt increased in proportion to ΔL; with larger releases Δt increased more than proportionally to ΔL. A nonlinear relationship as Limulus muscle fibers displayed in the slack test was also reported from experi-

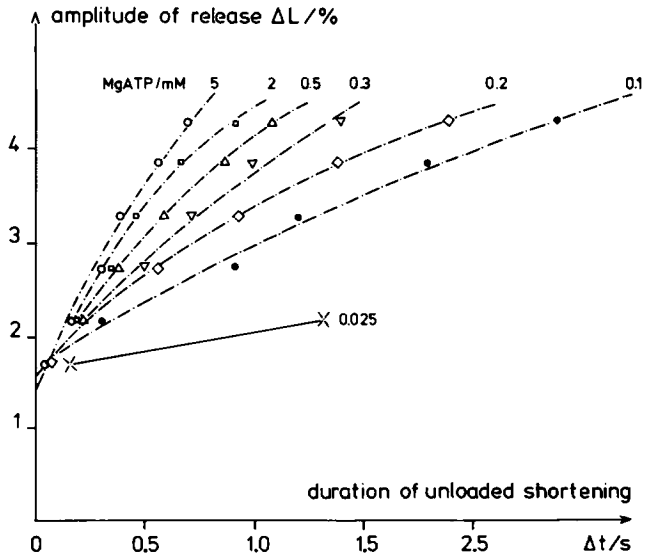


FIGURE 5 Relationship of length changes ΔL to duration of unloaded shortening Δt at various MgATP concentrations. Range of MgATP concentration 5, 2, 0.5, 0.3, 0.2, 0.1, and 0.025 mM as indicated. ΔL expressed as percentage of L_i, pCA = 4.9. The dotted lines were calculated by best fits of a hyperbola. Same muscle specimen as in Figs. 3 and 4.

ments executed with force clamp techniques on skinned frog muscle (Ferenczi et al., 1984) and on skinned rabbit psoas muscle (Brenner, 1980). In smooth muscle preparations (*Taenia coli* of the guinea pig, Gagelmann and Güth, 1985) the nonlinearity of the $\Delta L - \Delta t$ relation was more pronounced than in Limulus muscle. For a detailed discussion of this phenomenon see Brenner (1980) and Julian et al. (1986).

The velocities of unloaded shortening at the various MgATP concentrations were calculated extrapolating the derivative of the $\Delta L - \Delta t$ function to zero time. At low MgATP concentrations, the shortening velocity was low, and it increased as the MgATP concentration increased to reach a limiting value at ~1 mM MgATP (Fig. 6). The graph of Fig. 6 demonstrates that the Michaelis-Menten relation (Eq. 1) for saturation kinetics roughly fits the shortening velocity data. The Michaelis-Menten constant K_v was 95 ± 11 μM, and the maximum velocity of unloaded shortening at saturating MgATP concentration was 0.07 ± 0.02 muscle lengths s^{-1} (see also the Discussion).

Steady-State Rate of MgATP Hydrolysis

In the preceeding experiments the mechanical rate constants of the contractile system were determined with the muscle virtually unloaded. The following experiments were undertaken to determine the corresponding rate constants with the muscle isometrically contracting by measuring the rate of ATP cleavage for contracting (pCa 4.9) as well as for relaxed muscle fibers (pCa > 8). The measurements were carried out with the MgATP concentration in the bathing solution varying between 0.05 and 5 mM MgATP.

As shown in Fig. 7 (*open circles*), for increasing MgATP concentrations the rate of the MgATP hydrolysis

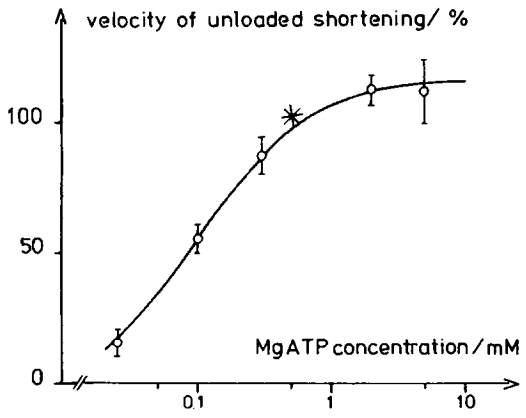


FIGURE 6 Effect of the MgATP concentration on the velocity of unloaded shortening. The ordinate is normalized to the value of velocity of unloaded shortening at 0.5 mM MgATP, 0.06 ± 0.02 muscle lengths s^{-1} (*). The solid line is the best fit of a Michaelis-Menten hyperbola with a K_v value of 95 ± 11 μM (three to six measurements per data point, SE values as indicated). L_i, 3–4.5 mm; cross-sectional area, 0.01–0.08 mm^2; sarcomere length, 7.5–9.5 μm; temperature, 13°C.

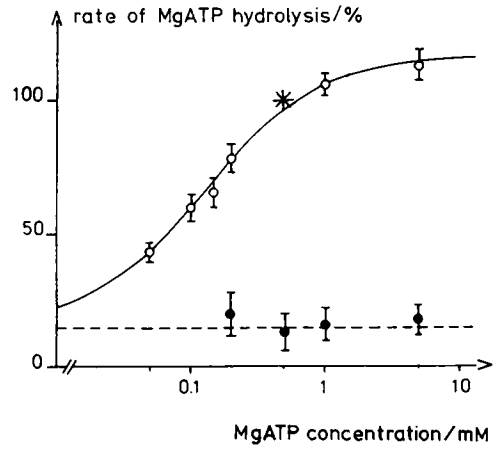


FIGURE 7 Rate of the MgATP hydrolysis in the presence (○) and absence (●) of free Ca^{++}, as functions of MgATP concentration. Rate of MgATP hydrolysis at 0.5 mM MgATP 9 ± 1 pmol s^{-1} per single fiber 10-mm long (100%, *); the graph of the rate of hydrolysis was best fit of the Michaelis-Menten relation with a K_a value of 129 ± 14 μM. Number of measurements, 11–13; SE values as indicated. L_i, 4–6 mm; cross-sectional area, 0.01–0.08 mm^2; sarcomere length, 7.5–9.5 μm; temperature, 22°C.

of isometrically contracting muscle fibers increased in a way that was best fit by a hyperbola based on Eq. 1. The maximum rate of MgATP hydrolysis, extrapolated from the fit at saturating MgATP concentration and related to the volume of a single fiber 10-mm long was 11 ± 1 pmol s^{-1}. With extrapolation towards zero MgATP concentration the hyperbola approximated the rate of MgATP hydrolysis of the fibers during relaxation which was independent of the MgATP concentration (Fig. 7, *solid circles*). The Michaelis-Menten constant K_a resulting from the fit was 129 ± 14 μM.

The molecular amount of myosin heads in 10 mm of Limulus muscle fiber was assumed to be ~10–20 pmol (cf. Wray et al., 1974). From this value and the maximum hydrolysis rate of 11 pmol s^{-1} per single fiber 10-mm long the maximum frequency of MgATP turnover of fibers exerting isometric contraction was estimated to be 0.5–1 s^{-1}.

The isometric tension generated by the fibers was determined simultaneously with each estimate of the MgATPase activity. The ratio of corresponding values of ATP hydrolyzed and tension developed is called the "tension cost" of force generation in the cross-bridge cycle. This ratio increased with increasing MgATP concentration (not illustrated).

DISCUSSION

Considerations on the Structure of Limulus Muscle

In all experiments, the sarcomere length of the muscle specimen was adjusted to 7.5–9.5 μm. In this range the length–tension relationship has its plateau of maximum

tension (Walcott and Dewey, 1980). It is discussed in relevant literature (cf. Dewey et al., 1982) whether or not the thick filaments of Limulus muscle shorten when the sarcomere length decreases below the rest length of 7 μm. Regardless of the contradictory views on this problem (Wray et al., 1974; Dewey et al., 1982) there is agreement that the thick filaments remain constant in length and cross-sectional area for sarcomere lengths between 7.2 and 11 μm. In this range the Limulus muscle is assumed to develop tension by a sliding filament mechanism.

Relation to Other Studies

Dependence of Shortening Velocity on MgATP Concentration. The roughly hyperbolic dependence of the velocity of unloaded shortening on the MgATP concentration observed in Limulus muscle fibers was also found for mechanically skinned frog semitendinosus muscle (Ferenczi et al., 1984) and for glycerinated rabbit psoas muscle (Cooke and Bialek, 1979). The value of the Michaelis-Menten constant K_v of the rabbit muscle (125 μM, 10°C, pH 7) agrees closely with the K_v value of the Limulus muscle (95 μM); the corresponding K_v value of the frog muscle is about four times higher (470 μM, 2°C, pH 7.1).

The estimation of the detachment rate of the cross-bridges is model dependent as discussed in detail by Eisenberg and Greene (1980). Adopting the model assumptions proposed by Ferenczi et al. (1984), we assume that the maximum speed of shortening can be related to the rate of cross-bridge detachment and that cross-bridges produce force during shortening over a distance of ~10 nm (Huxley, 1957; Huxley and Simmons, 1971). Then the speed of shortening of 0.07 muscle lengths s^{-1} relating to a sarcomere length of 8 μm yields a net detachment rate of 24 s^{-1}. The corresponding values of frog and rabbit muscle are about one order of magnitude higher than the Limulus value (frog semitendinosus: 267 s^{-1}, Ferenczi et al., 1984; rabbit psoas: ~300 s^{-1}, estimated from the data reported by Cooke and Bialek, 1979).

Michaelis-Menten Constant and Maximum Turnover Frequency of MgATPase. We found for Limulus muscle, that the steady-state rate of the MgATP hydrolysis measured under isometric conditions follows saturation kinetics with respect to the MgATP concentration. This result is in agreement with the findings for vertebrate skeletal muscle and insect flight muscle. The Michaelis-Menten constant of the MgATP hydrolysis of Limulus muscle (K_a = 129 μM) nearly equals the K_a values of glycerinated Lethocerus flight muscle (K_a = 140 μM, 20°C, pH 7.6; Kuhn et al., 1985), but it is about six times the K_a value of skinned frog semitendinosus muscle (K_a = 20 μM, 2°C, pH 7.1; Sleep, cited by Ferenczi et al., 1984) and of skinned rabbit psoas muscle (K_a = 17 μM, 15°C, pH 7; Glyn and Sleep, 1985).

The maximum turnover frequencies of the isometric ATPase activity are of the same order of magnitude in the glycerinated muscle fibers of the various species investigated. The isometric turnover frequencies are 0.5–1 s^{-1} in Limulus muscle, 1.2 s^{-1} in Lethocerus dorsal longitudinal muscle (Kuhn et al., 1985), 1.5 s^{-1} in frog semitendinosus muscle (Ferenczi et al., 1982), and 1.8 s^{-1} in rabbit psoas muscle (Glyn and Sleep, 1985).

It is interesting to compare the isometric ATP turnover frequencies with the ATPase activities studied in vitro in purified actin and myosin or in myofibrils. In frog, rabbit, and Lethocerus actomyosin, the in vitro ATP turnover frequencies are about five to eight times higher (pH 7.5, 25°C; Lehman and Szent-Györgyi, 1975) than the values of the corresponding muscle fibers held under isometric tension. In Limulus muscle, turnover frequencies estimated from the in vitro actomyosin ATPase activity, from the in vitro myofibrillar ATPase activity (Lehman and Szent-Györgyi, 1975), and from the isometric ATPase activity do not differ significantly from each other.

Bárány (1967) discussed a possible proportionality between the ATPase activities measured in vitro or in myofibrils of various myosins and the velocities of unloaded shortening of the corresponding muscle fibers. The velocities of unloaded shortening of Limulus, frog, and rabbit muscle fibers and the respective in vitro ATP turnover frequencies are roughly correlated in this way. The ATP turnover frequencies during isometric contraction of these muscles, however, do not correlate with the in vitro ATPase activities or with the shortening velocities.

Thus, during unloaded shortening the rate constants of cross-bridge detachment from actin resemble more or less the turnover frequencies of the ATP hydrolysis of actomyosin in solution (cf. Huxley, 1980). When the muscle fibers are held under isometric conditions, however, the reaction rates are strongly influenced by the constraints imposed on the elastic elements of the cross-bridges.

Effect of MgATP Concentration on Isometric Tension. At a pCa = 4.9 and a MgATP concentration of 5 mM, the isometric tension of skinned Limulus muscle fibers was ~20% of the maximum value measured in intact fibers (340 kN m^{-2}; Walcott and Dewey, 1980). Such difference is usually attributed to the swelling of the fibers that occurs when the surface membrane is removed (Maughan and Godt, 1979). Compared to frog muscle, in which the isometric tension exerted by skinned fibers was ~50% that of intact fibers (2°C, pH 7.1; Ferenczi et al., 1984), the loss of tension of glycerinated Limulus muscle is noticeably high.

The accumulation of inorganic phosphate within the fibers during Ca-activation seems not to be the cause for the low levels of isometric tension (Leonhardt et al., 1986). In recent experiments, we have tested the influence of the Ca^{++}, Mg^{++}, and H^+ concentration on the contraction of the Limulus muscle; it seems that the conditions of activation used for the experiments described in this paper were not maximal (Blankenbach, personal communication).

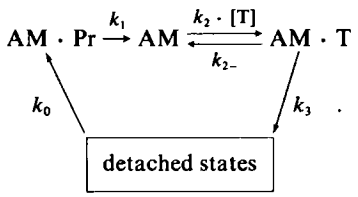
A similar dependence of the isometric tension on the MgATP concentration as illustrated in Fig. 2 for Limulus muscle fibers was reported for muscles from other species (rabbit psoas: Cooke and Bialek, 1979; frog semitendinosus: Ferenczi et al., 1984); (*a*) the Michaelis-Menten constants of the tension rise versus rising MgATP concentration of 2.3 μM for rabbit and of 1 μM for frog muscle are within the same order of magnitude as the constant K_{M1} = 7.5 μM of the Limulus muscle fibers; (*b*) the maximum of isometric tension is reached at concentrations of 50–100 μM MgATP; (*c*) when the MgATP concentration is raised to 1–5 mM, the tension values decrease to ~20–30% of the maximum value.

Slightly differing results were reported from other groups: Cox and Kawai (1981) found for chemically skinned rabbit psoas muscle (pH 7.0, 20°C) that the maximum tension was attained at concentrations of 0.3–1.0 mM MgATP. Under rigor conditions, the preparations of mammalian cardiac and insect flight muscle fibers exhibit a plateau of maximum tension; the tension decreases as the MgATP concentration is raised to millimolar levels (rat cardiac muscle: pH 7.0, 20°C, Best et al., 1977; Lethocerus flight muscle: pH 7.6, 20°C, Kuhn et al., 1985; pH 6.8, 17°C, Wilson and White, 1983).

We agree with the arguments brought forward by Ferenczi et al., (1984) that there exists no complete and satisfactory explanation of the dependence of isometric tension on the MgATP concentration (for theories cf. Ferenczi, 1979; Cooke and Bialek, 1979; Wilson and White, 1983). We expect that further experiments will be needed before a theoretical treatment of the induction of rigor states by lowered MgATP concentration can be completed.

Molecular Interpretation of the Results

Biochemical Pathway of MgATP Hydrolysis. The essential features of the ATP hydrolysis deduced from transient kinetic experiments using actin and myosin in solution have been reviewed by Taylor (1979), Eisenberg and Greene (1980), and Geeves et al. (1984). The interpretation of our results is based on a very simplified scheme of biochemical reactions which regards only four particular steps between actin (A), myosin heads (M), ATP (T), and the products of hydrolysis (Pr) depicted in Eq. 3:



(3)

The interdependence between the mechanical constraints that exist within the cross-bridges bound to the actin sites, and the chemical kinetics of the ATPase cycle in muscle fibers is specified in the theories of Huxley and Simmons (1971), Julian et al. (1973), Eisenberg and Greene (1980), or Harrington (1971). It is the purpose of this section to determine the reaction rates k_i of the simplified scheme Eq. 3 and to discuss their dependence on the binding position of the cross-bridges. The rate constants of "pushing" cross-bridges are termed $k_i^{(-)}$, and those of "pulling" cross-bridges are termed $k_i^{(+)}$.

Rate Constants $k_i^{(-)}$ of Pushing Cross-Bridges. Fig. 6 shows that the dependence of the shortening velocity of the MgATP concentration fits Eq. 1 based on Michaelis-Menten kinetics. An alternative interpretation is obtained by an analysis of the kinetic pathway postulated by the biochemical scheme Eq. 3 following closely the Huxley (1957) model of shortening velocity. Ferenczi et al. (1984) argued that, under these assumptions, the step of attachment will not markedly influence the velocity of unloaded shortening. The chemical rate equations of the steps 1–3 yield the following relations between the rate constants r_i for pushing cross-bridges and the MgATP concentration [T]:

$$r_0 = k_1^{(-)} \tag{4}$$

and

$$r^2 - r \cdot (k_2^{(-)} \cdot [T] + k_{2-}^{(-)} + k_3^{(-)}) + k_3^{(-)} \cdot k_2^{(-)} \cdot [T] = 0, \tag{5}$$

provided that $k_1^{(-)} < k_3^{(-)}$, the curve of the slower rate constants r (Eq. 5) intercepts the curve of the rate constant r_0 (Eq. 4), which does not depend on the substrate concentration. The MgATP concentration of this point of interception marks an abrupt change in the time course of the reaction cycle: At low MgATP concentrations, the shortening velocity is limited by the rate of substrate binding to the actomyosin. At high MgATP concentrations, it is limited by the product release (cf. also Ferenczi et al., 1984).

The rate constant $k_1^{(-)} = 24\ s^{-1}$ was determined from the values of the velocity of unloaded shortening at high MgATP concentrations (2–5 mM MgATP, Fig. 6). In vitro studies on myosins from slow muscles exhibit rate constants of the dissociation of ADP from the ternary AM·ADP complexes, which are within the same order of magnitude as our $k_1^{(-)}$ value of the Limulus muscle (Marston and Taylor, 1980; Siemankowski and White, 1984).

The rate constants $k_{2-}^{(-)} = 350\ s^{-1}$, $k_2^{(-)} = 10^6\ M^{-1}\ s^{-1}$, and $k_3^{(-)} = 30\ s^{-1}$ were obtained from the best fit of Eq. 5 to the velocities of unloaded shortening observed at MgATP concentrations equal to or lower than 0.5 mM (Fig. 6). These Limulus rate constants are of the same order of magnitude as the corresponding reaction rates found in biochemical studies on slow myosins (Marston and Taylor, 1980). With these $k_2^{(-)}$ and $k_{2-}^{(-)}$ values, the affinity constant for MgATP binding to the AM complexes was calculated to $K_2 = 3 \cdot 10^3\ M^{-1}$, which is comparable to the

corresponding values determined from in vitro studies (cf. Geeves et al., 1984).

Under steady-state conditions and at saturating MgATP concentrations, the kinetics of the steps AM · Pr → AM and AM · T → [detached states] determine one after the other the detachment rate of the cross-bridges from the actin; the apparent rate constant of detachment is then given by the relation:

$$k_d = \frac{k_1 \cdot k_3}{k_1 + k_3}, \tag{6}$$

which yielded $k_d^{(-)} = 8.6\ s^{-1}$ with the $k_1^{(-)}$ and $k_3^{(-)}$ values substituted.

Rate Constants $k_i^{(+)}$ of Force Generating Cross-Bridges. Provided that the steady-state cycling rate a_{max} of the isometrically contracted fibers is limited by an attachment rate constant k_0 and a detachment rate constant $k_d^{(+)}$ (Huxley, 1957; Kuhn et al., 1985; Brenner, 1986), then a_{max} is given by

$$a_{max} = \frac{k_0 \cdot k_d^{(+)}}{k_0 + k_d^{(+)}}. \tag{7}$$

For the Limulus muscle fibers, the rate constant of cross-bridge reattachment, $k_0 + k_d^{(+)} = 4\ s^{-1}$, was determined by Kulik (1986) using the technique of transient tension responses to quick length changes (cf. Huxley and Simmons, 1971). Since $a_{max} = 1\ s^{-1}$, the rate constants of detachment and attachment could be evaluated to $k_d^{(+)} = 2\ s^{-1}$ and $k_0 = 2\ s^{-1}$. Thus in Limulus fibers, the apparent rate constant of cross-bridges detaching from actin is about four or five times higher for pushing cross-bridges (unloaded shortening) than for pulling cross-bridges (isometric state). In frog muscle fibers, the corresponding ratio of the apparent detachment rate constants of pushing and of pulling cross-bridges is at least 20:1 (Huxley, 1957; Ferenczi et al., 1984).

In analogy to Glyn and Sleep (1985), the apparent biomolecular rate constant $k_{2app}^{(+)}$ of MgATP binding to the AM complexes can be derived from the relation between turnover frequency of ATPase activity and MgATP concentration (Fig. 7); it was calculated to $k_{2app}^{(+)} = 8 \cdot 10^3\ M^{-1}\ s^{-1}$. A comparison of $k_2^{(-)}$ and $k_{2app}^{(+)}$ shows that in slow Limulus muscle, the mechanical constraints reduce the apparent bimolecular rate constant k_2 by a factor of ~100 when the cross-bridges move from pushing to pulling binding positions. A comparable dependence of the bimolecular rate constant $k_{2app}^{(+)}$ on cross-bridge constraints was not discussed for fast frog muscle fibers (Ferenczi et al., 1984).

CONCLUSIONS

During unloaded shortening at physiological MgATP concentrations, the release of the hydrolytic products from the actomyosin complex appears to be the step of the cross-bridge cycle that limits the shortening velocity in slow Limulus muscle. In this respect, Limulus muscle is comparable with fast frog muscle (cf. also Siemankowski and White, 1984). Our experiments suggest that in Limulus muscle the rate constant of detachment depends on the binding position ("pushing" or "pulling") of the cross-bridge, and that the step of MgATP-binding to rigor linkages depends markedly on the strain within the fiber. These findings support the view that mechanical constraints affect mainly the steps in which strong-binding states of the cross-bridge cycle are involved. The current model is relatively simple, but it may be the base for comparing the results obtained from transient tension responses and from the dynamic measurements of the ATPase activity in muscle fibers with the complex kinetics of the cross-bridge cycle as revealed by in vitro studies.

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