Elementary Steps of the Cross-Bridge Cycle in Bovine Myocardium with and without Regulatory Proteins

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ABSTRACT The role of regulatory proteins in the elementary steps of the cross-bridge cycle in bovine myocardium was investigated. The thin filament was selectively removed by gelsolin and the actin filament was reconstituted without tropomyosin or troponin. Further reconstitution was achieved by adding tropomyosin and troponin. The effects of MgATP and phosphate (Pi) on the rate constants of exponential processes were studied in control, actin filament-reconstituted, and thin filament-reconstituted myocardium at pCa ≤4.66, pH 7.00, 25°C. In control myocardium, the MgATP association constant was 9.1 ± 1.3 mM^{-1}, and the Pi association constant 0.14 ± 0.04 mM^{-1}. The equilibrium constant of the cross-bridge detachment step was 2.6 ± 0.4, and the equilibrium constant of the force generation step was 0.59 ± 0.04. In actin filament-reconstituted myocardium without regulatory proteins, the MgATP association constant was approximately the same, and the Pi association constant increased to 2.8×. The equilibrium constant of cross-bridge detachment decreased to 0.2×, but the equilibrium constant of the force generation step increased to 4×. These kinetic constants regained control values after reconstitution of the thin filament. These results indicate that tension/cross-bridge in the presence of regulatory proteins is ~1.5–1.7× of that in the absence of regulatory proteins. These results further indicate that regulatory proteins promote detachment of cross-bridges.

INTRODUCTION

Striated muscle is a complex system that consists of impulse generation and propagation mechanisms, Ca^{2+} release and uptake mechanisms, Ca^{2+}-sensing and signal-transmission mechanisms, and actomyosin-force generation mechanisms. This complexity makes it difficult to interpret the experimental results when carried out in intact preparations, if the purpose was to understand the cross-bridge mechanisms of force generation. The introduction of chemically skinned (Szent-Györgyi, 1951) and mechanically skinned (Natori, 1954) fibers in which the sarcolemmal barrier was removed was a step forward to overcoming this problem. With the loss of the sarcolemmal barrier, chemical perturbation can be readily applied to the skinned fibers. In addition, the use of a detergent minimizes the Ca^{2+}-uptake mechanism of the sarcoplasmic reticulum and ATP-regenerating mechanism of mitochondria, thus further simplifying the system. The detergent-treated skinned fibers still possess the Ca^{2+}-regulatory system, tropomyosin (Tm) and troponin (Tn) (Endo et al., 1970), which is known to alter the actomyosin kinetics (Murray et al., 1975; Zhao et al., 1996). More recently, investigators have demonstrated with the in vitro motility assay that both gliding speed and force increase by the addition of the regulatory proteins (Gordon et al., 1998; Van Buren et al., 1999; Bing et al., 2000a, b; Homsher et al., 2000). It is desirable to demonstrate these points in physiological ionic strength in skinned fibers, in which force generation capability is maintained, because the in vitro assay is typically carried out at low ionic strength (~50 mM). The low ionic strength enhances the ionic interaction, whereas the major mechanism of force generation may rely on hydrophobic interaction (Kodama, 1985; Rayment et al., 1993; Zhao and Kawai, 1994). To study the influence of regulatory proteins on the cross-bridge kinetics in muscle fibers, it is necessary to modify the regulatory component of the contractile apparatus without interfering with the force-generating capability. However, the extraction-reconstitution method has been limited to small molecules, such as troponin C (Brandt et al., 1990; Zhao et al., 1996), troponin I (Hatanaka and Ohtsuki, 1992; Strauss et al., 1992), and myosin light chain 2 (Hofmann et al., 1990), that play accessory roles for contraction. A new approach using transgenic mice to generate tropomyosin rich in β-isoform (Palmiter et al., 1996) is a similar line of work.

Recently, Fujita et al. (1996) have developed a method that can completely remove the thin filament and reconstitute it in bovine myocardium. In this method, the thin filament in the myocardium was selectively removed by treatment with gelsolin, an actin-severing enzyme, to result in a loss of contractility. The actin filament was reconstituted from purified G-actin under the polymerizing condition. The resulting myocardium was capable of generating tension that was insensitive to Ca^{2+}. Further reconstitution with regulatory proteins Tm and Tn fully recovered the Ca^{2+} sensitivity and isometric tension. A parallel experiment was performed on skeletal muscles which resulted in a formation of the actin filament, but reproducibility of

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TABLE 1 Basic solutions

Solution Symbols	K_2CaEGTA mM	K_2EGTA mM	Na_2MgATP mM	Na_2K_2ATP mM	$MgProp_2$ mM	Na_2CP mM	$K_{1.5}$Pi mM	NaProp mM	Kprop mM	NaN_3 mM	MOPS mM	BDM mM	CK U/ml
Rigor (Rg)	—	—	—	—	—	—	8	55	122.0	—	10	—	—
Relax (Rx)	—	6	2.2	5.0	—	—	8	41	74.5	—	10	40	—
5S0P	6	—	5.83	1.36	—	15	0	0.6	91.7	10	10	—	320
5S32P	6	—	5.7	1.36	—	15	32	0.9	17.3	10	10	—	320
0S8P	6	—	—	—	0.85	15	8	15	87.6	10	10	—	320
5S8P	6	—	5.8	1.36	—	15	8	0.7	73.1	10	10	—	320
−Ca	—	6	5.95	1.13	—	15	—	0.6	91.7	10	10	—	320

Abbreviations: Prop = propionate, CP = creatine phosphate, and CK = creatine kinase. pH of all solutions was adjusted to 7.00.

isometric tension was not as large as in myocardium, presumably because of the presence of nebulin and weak Z-line (Funatsu et al., 1994). In this report, we have combined the actin filament-reconstitution technique with the sinusoidal analysis technique, and the effect of MgATP and Pi concentrations on the rate constants of exponential processes was studied in myocardium both with and without regulatory proteins. Our results indicate that the role of regulatory proteins is to augment force supported by each cross-bridge.

MATERIALS AND METHODS

Chemicals and solutions

Creatine phosphate (Na_2CP), adenosine 5′-triphosphate (Na_2H_2ATP), 3-[*N*-morpholino] propane sulfonic acid (MOPS), and ethylene glycol bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′ tetraacetic acid (H_4EGTA) were purchased from Sigma Chemical (St. Louis, MO); Triton-X100, (Fisher Scientific, Hanover Park, IL); $CaCO_3$, $Mg(OH)_2$, NaOH, KOH, KH_2PO_4, K_2HPO_4, NaN_3, and propionic (Prop) acid were from Fisher Scientific; and creatine kinase (CK) was from Boehringer Mannheim (Indianapolis, IN).

The compositions of each solution used are shown in Table 1. Experimental solutions are designated by *m*S*n*P, where *m* represents the millimolar concentration of $MgATP^{2-}$ (S), and *n* represents that of phosphate (P, Pi): for example, the 5S8P solution contained 5 mM $MgATP^{2-}$ and 8 mM Pi. The 5S8P solution is also called the standard activating solution. The pCa of all activating solutions was 4.35–4.66, pH was adjusted to 7.00, the Mg^{2+} concentration to 0.5 mM, the total Na to 55 mM, and ionic strength to 200 mM. All activating solutions contained 320 units/ml CK (0.64 mg/ml), and 10 mM NaN_3 to suppress mitochondrial ATPase. EGTA, CaEGTA, and Pi were added as neutral K salts; MgATP and CP as neutral Na salts; and free ATP as $Na_2K_{1.7}$ATP (neutral salt). Individual concentrations of ionic species were calculated with our computer program using the following association constants (log values at pH 7.0): CaEGTA, 6.28; MgEGTA, 1.61; CaATP, 3.70; MgATP, 4.00; CaCP, 1.15; MgCP, 1.30.

Muscle bundles and proteins

Bovine hearts were obtained from a slaughterhouse and immediately cooled with crushed ice. The muscle bundles (~2 mm in diameter and 10 mm in length) were excised from a straight portion of left ventricular papillary muscles and incubated in the Na-skinning solution containing (mM) 2.0 dithiothreitol (DTT), 30 2,3-butanedione 2-monoxime (BDM), 10 Na_2H_2EGTA, 5.0 Na_2H_2ATP, 2.0 Na_2MgATP, 122 Na propionate (Prop), and 10 MOPS (pH 7.0) for 3 h at 0°C. The Na-skinning solution was used to minimize initial contraction. For further skinning, the solution was replaced with K-skinning solution containing (mM) 2.0 DTT, 30 BDM, 10 K_2H_2EGTA, 5.0 Na_2H_2ATP, 2.0 Na_2MgATP, 122 KProp, and 10 MOPS (pH 7.0), and stored overnight at 0°C. BDM and EGTA were used to minimize force generation. The solution was further replaced with a solution containing 50% (v/v) glycerol, 2.0 DTT, 30 BDM, 10 K_2H_2EGTA, 5.0 Na_2H_2ATP, 2.0 Na_2MgATP, 122 KProp, and 10 MOPS (pH 7.0) and stored at 0°C. The solution was replaced once again the next day, and the muscle bundles were stored in a freezer (−20°C).

Actin was extracted from acetone powder (Kondo and Ishiwata, 1976) of rabbit white skeletal muscles according to the method of Spudich and Watt (1971). We used rabbit skeletal actin for reconstitution because its sequence is almost identical to bovine cardiac actin with four minor substitutions (cardiac → skeletal: D2E, E3D, L298M, S357T) (Vandekerckhove and Weber, 1979). Purified G-actin was stored at 0°C and used within 2 weeks of extraction. The tropomyosin (Tm)-troponin (Tn) complex (nTm) was prepared from bovine cardiac muscle according to the method of Ebashi et al. (1968). Bovine plasma gelsolin was prepared according to the method of Kurokawa et al. (1990).

Selective removal and reconstitution of actin filament were performed as previously described (Fujita et al., 1996; Fujita and Ishiwata, 1998, 1999; Ishiwata et al., 1998). Particular care was taken to regulate the temperature and time of actin filament reconstitution protocol to prevent excessive elongation of the actin filament (Fujita and Ishiwata, 1998, 1999; Ishiwata et al., 1998) as seen in the initial report (Fujita et al., 1996).

Experimental procedure and deduction of kinetic constants

A strip of bovine myocardium was removed from the skinned muscle bundle and both ends were attached to a stainless steel wire (diameter: 210 μm) with nail polish, one end of which was attached to a tension transducer and the other to a length driver. The myocardium was stretched until a small passive tension was observed. At this point, the average sarcomere length was 2.0 μm, and it ranged 1.9–2.1 μm as determined by confocal microscopy after staining with rhodamine-conjugated phalloidin. The length (L_0 ~2 mm) of the myocardium was determined by measuring the end-to-end distance. The diameter was measured under a dissection microscope (20×). The diameter averaged 110 ± 2 μm (n = 73, ± SE). Myocardium was chemically skinned further in relaxing solution containing 1% (v/v) Triton-X100 for 20 min. Triton-X100 was washed by relaxing solution (Rx) before experiments.

Three muscle models (control, actin filament-reconstituted, and thin filament-reconstituted myocardium) were maximally activated in the presence of Ca^{2+} in a temperature-controlled bath at 25°C for subsequent experiments. The 0.25% L_0 peak-to-peak sinusoidal wave at 18 discrete frequencies (f: 0.13–100 Hz) was digitally synthesized in a personal computer with 386 CPU (Industrial Computer Source, San Diego, CA) that controlled the length driver via a 14-bit digital/analog converter. Tension and length signals were simultaneously digitized by two 16-bit analog/digital converters and complex modulus data $Y(f)$ were calculated as the ratio of force change and length change in the frequency domain. The

complex modulus data obtained during relaxation were subtracted to correct for the irrelevant pickup at high frequencies. The data were further corrected by using the rigor response. The complex modulus data were resolved into two exponential processes (B and C) by fitting the data to Eq. 1 (Kawai and Brandt, 1980; Wannenburg et al., 2000):

$$\text{Process B} \qquad \text{Process C}$$

$$Y(f) = H - B/(1 + b/fi) + C/(1 + c/fi) \qquad (1)$$

where $i = \sqrt{-1}$. Lowercase letters b and c represent the characteristic frequencies of the respective processes, and uppercase letters B and C represent their respective magnitudes. $2\pi b$ and $2\pi c$ are the apparent rate constants of the respective processes. Process B is a low-frequency exponential delay ($b \sim 1$ Hz) at which muscle generates oscillatory work. Process C is a high frequency exponential advance ($c \sim 4$ Hz) at which the muscle absorbs work from the length driver. Stiffness was calculated as $Y_\infty = H - B + C$. Process B corresponds to "phase 3," process C corresponds to "phase 2," and Y_∞ corresponds to "phase 1" of tension transients in response to a step-length change (Huxley and Simmons, 1971; Heinl et al., 1974). An implicit assumption of the transient analysis in response to step or sinusoidal length change is that the rate constants of elementary steps are strain-sensitive (Huxley and Simmons, 1971; Kawai and Brandt, 1980; Kawai and Zhao, 1993), so that a length change causes an instability in the cross-bridge cycle. A gradual transition to the new steady state is observed as tension transients in step analysis and exponential processes in sinusoidal analysis. In relaxed and rigor muscle fibers, these transients or exponential processes are absent, indicating that they are the results of cycling cross-bridges. Details of the sinusoidal analysis technique have been published (Kawai and Brandt, 1980).

Sodium dodecyl sulfate (SDS)-gel electrophoresis

Myocardium at each step of reconstitution was pooled and dissolved in a sample diluting buffer (2% SDS, 25% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) and heated for 3 min at 90°C. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970) with an 8–16% linear gradient running gel and a 4% stacking gel (Bio-Rad, Hercules, CA). Protein was stained using Coomassie brilliant blue R-250.

RESULTS

Removal of the thin filament and reconstitution of the actin filament and the thin filament in myocardium

Fig. 1 shows a result from SDS-PAGE of control, gelsolin-treated, actin filament-reconstituted, and thin filament-reconstituted myocardium. In gelsolin treated myocardium (*lane 2*), the amount of actin, TnT, Tm, and TnI was markedly reduced or absent compared with the control myocardium (*lane 1*), whereas the amount of myosin heavy chain (HC), α-actinin (α-Ac), myosin light chain 1 (LC_1), myosin light chain 2 (LC_2) did not change much. A new band is seen in lane 2 below α-Ac, and this is gelsolin. In the actin filament-reconstituted myocardium (*lane 3*), actin recovery was nearly equal to that of the control, whereas Tm, TnI, and TnT are absent. After reconstitution with regulatory proteins (*lane 4*), the amount of Tm, TnI, and TnT recovered to a level similar to the control. The calculated density ratio of actin/LC_1 of gelsolin-treated myocardium was 14% of control myocardium, but this value is inflated because the actin band in control myocardium is saturated because of overloading. Thus, the amount of remaining actin in gelsolin-treated myocardium must have been <14%. The remaining actin is essential for subsequent elongation of the actin filament. We could not resolve the TnC band because cardiac TnC appears close to LC_2, and because the staining of TnC is weak compared with LC_2 with the method we used. There is a weak band between two light chains, but its origin is unknown.

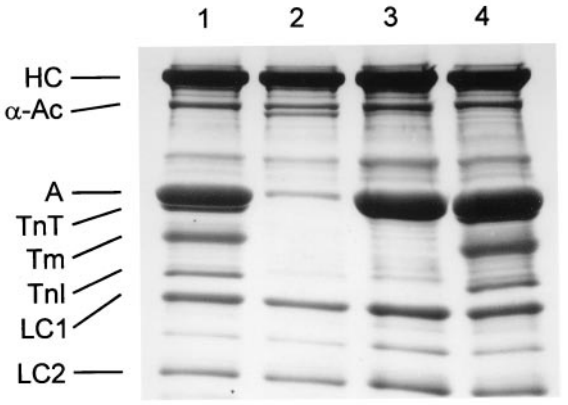


FIGURE 1 SDS-PAGE of control (*lane 1*), gelsolin-treated (*lane 2*), actin filament-reconstituted (*lane 3*), and thin filament-reconstituted (*lane 4*) myocardium. See Fig. 2 for detail of the treatments.

Fig. 2 shows a slow pen trace of isometric tension at each step of removal and reconstitution of the thin filament. Myocardium was initially tested by the Ca^{2+}-activating solution (5S0P) without Pi (Fig. 2 *A*). Then at G, the myocardium was treated by the solution that contained (mM) 117 KCl, 4.25 $MgCl_2$ (2.2 free Mg^{2+}), 2.2 Na_2H_2ATP (2.0 $MgATP^{2-}$), 2.0 H_4EGTA, 20 MOPS (pH 7.0), 2.0 $CaCl_2$, 40 BDM, and 0.3 mg/ml gelsolin at 2°C for 100 min to selectively remove the thin filament. BDM was used to suppress tension development during gelsolin treatment that requires Ca^{2+}. There was no active tension development when tested with the 5S0P solution (pCa 4.66) after gelsolin treatment (Fig. 2 *B*). At Ac, the myocardium was treated by the actin-polymerizing solution that contained (mM) 80 KI, 4.0 $MgCl_2$, 4.0 ATP, 4.0 EGTA, 40 BDM, 20 K-phosphate (pH 7.0), and 1.0 mg/ml G-actin at 2°C to reconstitute the actin filament. The actin-polymerizing solution was replaced every 7 min to avoid nucleation of actin. Similarly, KI was used to deter nucleation. After a total of 28 min (7 min × 4) of actin polymerizing treatment (Ac), this particular myocardium developed ~65% of the control tension (Fig. 2 *C*). Average active tension after reconstitution of the actin filament was 69 ± 5% ($n = 26$). In the actin filament-reconstituted myocardium, isometric tension developed in the solution that lacked Ca^{2+}, indicating the absence of regulatory proteins (Fujita et al., 1996).

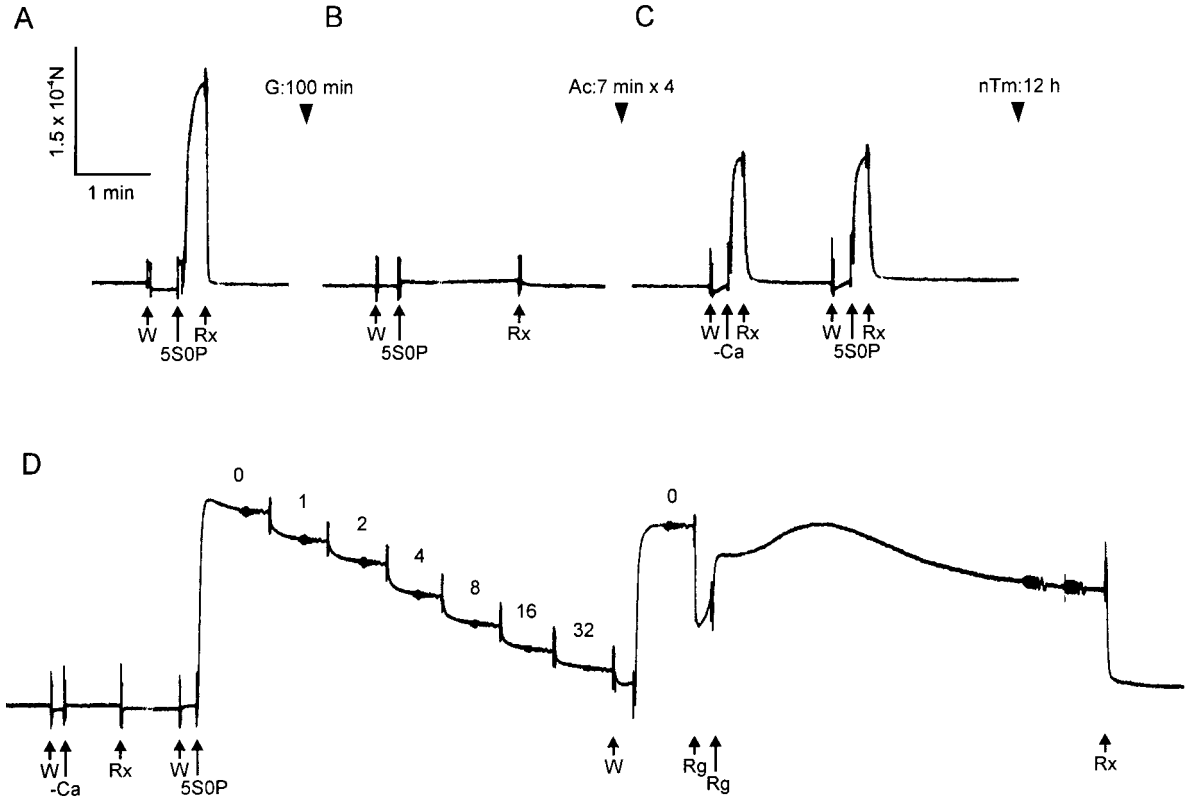


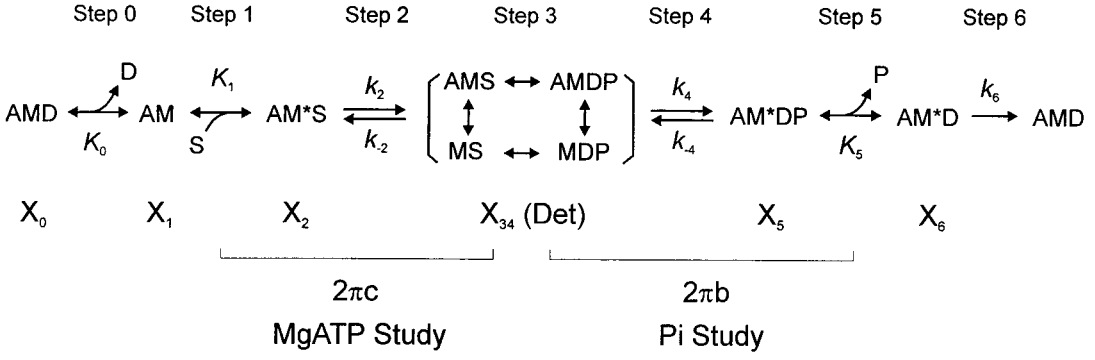
FIGURE 2 A slow pen trace of isometric tension at each step of removal and reconstitution of the thin filament. (*A*) Control myocardium; (*B*) After gelsolin treatment for 100 min (G); (*C*) After actin filament-reconstitution (Ac); (*D*) After thin filament-reconstitution (nTm). Control myocardium was first activated in 5S0P solution (pCa 4.66) at 25°C (*A*). Before activation, myocardium was immersed in the same 5S0P solution at 0°C to wash out BDM and Pi (W). Myocardium did not develop active tension in W because of the low temperature. After gelsolin treatment, myocardium was immersed in 5S0P solution at 25°C to confirm the removal of thin filament (*B*). After reconstitution of actin filament (Ac), myocardium was immersed in the solution without Ca^{2+} (−Ca) then the solution with Ca^{2+} (5S0P) at 25°C to confirm the absence of the regulatory system. The amount of isometric tension developed did not depend on Ca^{2+} (*C*). After reconstitution of regulatory proteins (nTm), active tension did not develop without Ca^{2+}, but it developed with Ca^{2+} (*D*). In *D*, the complex modulus data were collected at seven different Pi concentrations (0–32 mM), and the initial activation at 0 mM Pi was repeated to detect any deterioration in the preparation. The preparation was discarded when a >20% tension decrease was noticed. The relaxation was obtained in the solution containing 40 mM BDM (Rx) at 0°C. All activations including rigor were performed at 25°C. All records in this figure were taken sequentially from the same myocardium. Numbers above tension trace in (*D*) indicate the mM Pi concentration.

As seen in Fig. 2 *C*, isometric tension did not depend on Ca^{2+} in the actin filament-reconstituted myocardium. Relaxation was achieved by immersing the myocardium in the solution containing 40 mM BDM (Rx) at 0°C. To reconstitute the thin filament, the actin filament-reconstituted myocardium was then immersed in the Rx solution containing 4 mg/ml Tm-Tn complex for 12 h at 0°C (nTm). Care was taken so that the volume of the solution did not change because of evaporation or condensation. After reconstitution of regulatory proteins with nTm, the myocardium regained the Ca^{2+} sensitivity. It did not develop tension in the absence of Ca^{2+}, and did develop active tension in the presence of Ca^{2+} (Fig. 2 *D*). We infer from this observation that the reconstitution of the thin filament was complete. Isometric tension after reconstitution of regulatory proteins was 105% of that of the control in this particular myocardium (compare Fig. 2, *A* and *D*), and averaged 107 ± 4% (n = 26). In other words, the reconstitution of Tm and Tn augmented active tension by ~50%. Thus, it can be concluded that higher force is supported if Tm and Tn are present under the experimental conditions we examined.

Effect of MgATP on exponential process C

MgATP and Pi studies were performed to investigate whether the larger force, observed in the presence of Tm and Tn but not in their absence, is related to larger force/cross-bridge or to the larger number of cross-bridges in force-generating states. The effect of MgATP on exponential process C on control, actin filament-reconstituted, and thin filament-reconstituted myocardium (three muscle models) was studied in the range of 0.05–5.00 mM under the maximal Ca^{2+}-activating condition (pCa 4.35–4.66) in the presence of 8 mM Pi. The purpose was to characterize the elementary steps of MgATP binding and subsequent cross-

bridge detachment steps. 8 mM Pi was used because more cross-bridges are populated at AM, AMS, and Det states (scheme 1) than in its absence, hence characterization of transitions between these states is easier. The cross-bridge scheme is depicted in the following.



Scheme 1

where A = actin ± regulatory proteins, M = myosin, S = $MgATP^{2-}$, D = MgADP, and P = phosphate. Detached states (MS, MDP) and weakly attached states (AMS, AMDP) are lumped together and called the Det state. Two extreme MgATP solutions (0S8P and 5S8P, Table 1) were prepared and intermediate solutions were made by appropriately mixing the two solutions without affecting other ionic concentrations. Fig. 3 shows complex modulus $Y(f)$ in three muscle models activated at three different MgATP concentrations (0.1 mM, 0.5 mM, 5 mM). These plots are in general similar to those reported on other myocardial systems (Kawai et al., 1993; Zhao and Kawai, 1996, Wannenburg et al., 2000) in their shape and frequency response.

In cardiac muscles, there might be a concern that $2\pi b$ and $2\pi c$ are a part of the same process, because they are close together and only differ by the factor of ~3. A close examination of Fig. 3, *C*, *F*, and *I* indicates that there are at least two semicircles in the Nyquist plots: one going through the fourth quadrant, and the other going through the first quadrant. Note that, with an increase of frequency, the elastic modulus (Fig. 3, *A*, *D*, and *G*) decreases in the low-frequency range, and it increases at the high-frequency range. Also note that the viscous modulus (Fig. 3, *B*, *E*, and *H*) is negative in the low-frequency range, and it becomes positive at the high-frequency range. These shapes unambiguously justify the presence of two exponential processes and validate Eq. 1 as an approximation of the complex modulus data of activated myocardium.

The complex modulus data obtained were fitted to Eq. 1 to deduce the apparent rate constant $2\pi c$. The MgATP dependence on $2\pi c$ of control (○), actin filament-reconstituted (▲), and thin filament-reconstituted (□) myocardium is shown in Fig. 4 with SE error bars. The kinetic constants of elementary steps 1 and 2 of the cross-bridge cycle (scheme 1) were determined by fitting the MgATP dependence of $2\pi c$ to Eq. 2 (Kawai and Halvorson, 1991).

$$2\pi c = k_2K_1S/(1 + K_1S) + k_{-2} \quad (2)$$

where S denotes the concentration of $MgATP^{2-}$. When the three curves in Fig. 4 are compared, there is one obvious difference: the zero intercept (= k_{-2}) of ▲ is twice that of ○ and □. Other features of the curves, such as the half-saturation point (= $1/K_1$) and infinite ATP asymptote (= $k_2 + k_{-2}$) do not seem to be much different in the three curves. These features result in approximately $0.5 \times k_2$, $2 \times k_{-2}$, $0.25 \times K_2$, and little change in K_1 if regulatory proteins Tm and Tn are absent. The actual result of the data fitting is summarized in Table 2. As seen in Table 2, in control myocardium, the association constant of MgATP to cross-bridges (K_1) was 9.1 ± 1.3 mM^{-1} (± SE, n = 11). The forward rate constant of cross-bridge detachment step 2 (k_2) was 26.6 ± 1.2 s^{-1}. The backward rate constant (k_{-2}) was 12.1 ± 1.3 s^{-1}. The equilibrium constant K_2 (= k_2/k_{-2}) was 2.6 ± 0.4. In actin filament-reconstituted myocardium without regulatory proteins, K_1 slightly decreased to 0.95×; k_2 decreased to 0.50×; k_{-2} increased to 1.9×; and K_2 decreased to 0.23×. The kinetic constants of the thin filament-reconstituted myocardium were not different from the control myocardium (Table 2), indicating that the reconstitution was functionally complete.

Effect of phosphate (Pi) on exponential process B

To determine the kinetic constants associated with elementary steps 4 and 5, we studied the effect of Pi in the range of 0–32 mM on exponential process B in the three muscle models (Fig. 5). These studies were carried out under the maximal Ca^{2+}-activating condition (pCa 4.66) and in the presence of a saturating MgATP concentration (5 mM). Two Pi solutions, 5S0P and 5S32P, were initially prepared, and these were mixed appropriately to obtain intermediate

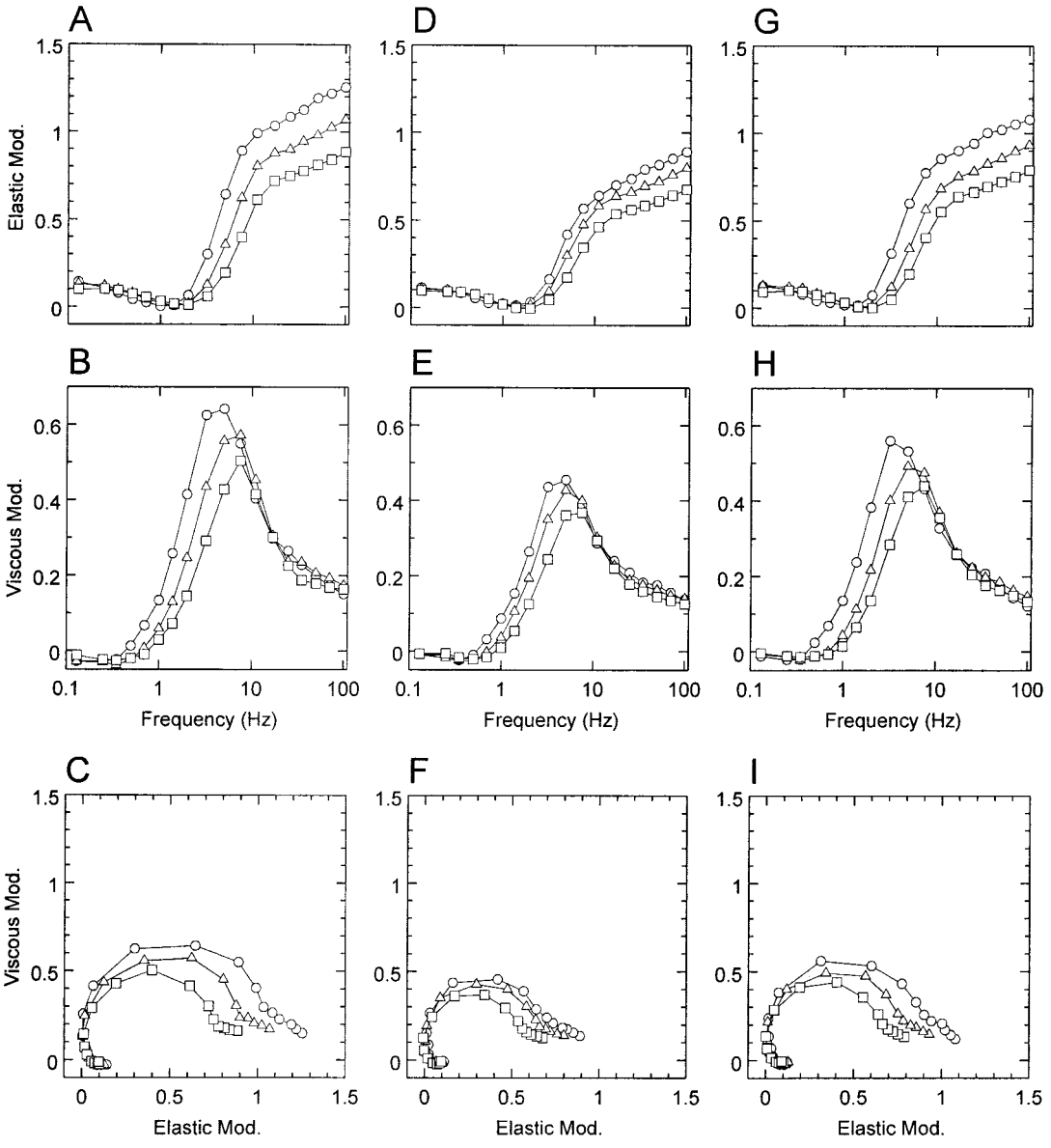


FIGURE 3 The effects of MgATP on the complex modulus $Y(f)$ in (*A–C*) control, (*D–F*) actin filament-reconstituted, and (*G–I*) thin filament-reconstituted myocardium. (*A*, *D*, and *G*) Elastic modulus vs. frequency. (*B*, *E*, and *H*) Viscous modulus vs. frequency. (*C*, *F*, and *I*) Data shown in Nyquist plot, which is a plot of elastic modulus (in abscissa) vs. viscous modulus (in ordinate). 0.1 mM MgATP (○), 0.5 mM MgATP (△), and 5 mM MgATP (□). The phosphate concentration was fixed to 8 mM. Peak-to-peak amplitude was 0.25% L_0. T_c (initial control tension) was 29 ± 3 kN/m^2 (n = 11) for control myocardium; 27 ± 3 kN/m^2 (n = 13) for actin filament-reconstituted myocardium; and 29 ± 3 kN/m^2 (n = 11) for thin filament-reconstituted myocardium. The units of elastic and viscous moduli are mN/m^2.

Pi solutions. The complex modulus data were fitted to Eq. 1 to obtain the apparent rate constant $2\pi b$. $2\pi b$ was then plotted against the Pi concentration and fitted to Eq. 3 (Kawai and Halvorson, 1991) to deduce the kinetic constants of elementary steps 4 and 5 of the cross-bridge cycle (Fig. 5).

$$2\pi b = \sigma k_4 + k_{-4}K_5P/(1 + K_5P) \quad (3)$$

where

$$\sigma = K_2K_1S/\{1 + (1 + K_2)K_1S\} \quad (4)$$

and P indicates the Pi concentration. K_1 and K_2 obtained from the MgATP study and S = 5 mM were used for calculation of σ. The results are summarized in Table 2. In the control myocardium, the rate constant of the force generation step (k_4) was 7.1 ± 0.6 s^{-1} (n = 10) and its reversal step (k_{-4}) was 12.6 ± 1.3 s^{-1}. The equilibrium

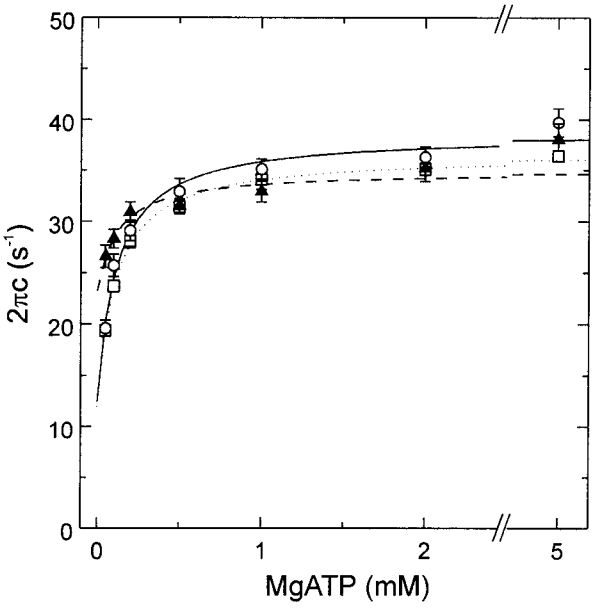


FIGURE 4 The rate constant $2\pi c$ is plotted as a function of MgATP concentration. (○ *and continuous line*) Bovine control myocardium (n = 11); (▲ *and dashed line*) actin filament-reconstituted myocardium (n = 7); (□ *and dotted line*) thin filament-reconstituted myocardium (n = 11). Error bars represent SE. Continuous curves are based on Eq. 2.

constant K_4 (= k_4/k_{-4}) of the force generation step was 0.59 ± 0.04, and the Pi association constant (K_5) was 0.14 ± 0.04 mM^{-1}. In actin filament-reconstituted myocardium, k_4 increased to 1.6×; k_{-4} decreased to 0.4×; K_4 increased to 4×; and K_5 increased to 2.8×. The kinetic constants of the thin filament-reconstituted myocardium were not significantly different from the control myocardium. Once again, this fact indicates that the reconstitution was functionally complete.

The endogenous Pi concentration in myocardium in the absence of added Pi

The Pi concentration in myocardium in the absence of added Pi is not zero. This is because Pi may be a contaminant in various phosphate compounds, particularly in Na_2CP, and because of continuous hydrolysis of ATP. One method of estimating the endogenous phosphate concentration (P_0) is to extrapolate $2\pi bB$ to the low Pi concentration, and to determine the Pi concentration in which $2\pi bB$ becomes zero (see Eq. 3 and Fig. 2 *C* of Kawai and Zhao, 1993). This is a negative value and equals $-P_0$. We found that there is no significant difference in the P_0 values for the three models of myocardium (control is 1.12 ± 0.25, n = 5; actin filament-reconstituted is 1.05 ± 0.26, n = 6; and thin filament-reconstituted is 1.08 ± 0.26, n = 15), and they averaged 1.08 ± 0.10 mM (n = 26). This endogenous Pi concentration has been taken into account in determining the kinetic constants of steps 4 and 5. This value compares with 0.6 mM in rabbit psoas fibers using the same method (Kawai and Halvorson, 1991), and 0.2 mM obtained using sucrose and the sucrose phosphorylase system to reduce the endogenous Pi (Pate and Cooke, 1989; Millar and Homsher, 1992).

Cross-bridge distribution

We calculated the steady-state distribution (probability) of cross-bridges in three muscle models by using Eq. 18 of Kawai and Halvorson (1991). The calculation was based on the equilibrium constants in Table 2 and on the standard activating condition (5 mM MgATP, 8 mM Pi). Fig. 6 shows the cross-bridge distribution obtained from the control (*white bars*), actin filament-reconstituted (*black bars*), and thin filament-reconstituted (*stippled bars*) myocardium. In all muscle models, the probability of cross-bridges in the AM state is ≤0.6% and not significantly populated. Cross-bridges are distributed by 13–44% in every other state. In the actin filament-reconstituted myocardium, both attached states AM*S and AM*DP increased to 1.8×, another attached state AM*D decreased to 0.7×, whereas the detached and weakly attached states (Det) decreased to 0.4×. In the thin filament-reconstituted myocardium, cross-bridge distributions regained the control values.

TABLE 2 The kinetic constants and the equilibrium constants of control, actin filament-reconstituted, and thin filament-reconstituted bovine myocardium

Kinetic Constants	Units	Control Myocardium	Actin Filament-Reconstituted Myocardium	Thin Filament-Reconstituted-Myocardium
K_1	mM^{-1}	9.1 ± 1.3 (11)	8.6 ± 2.5 (7)	9.9 ± 1.6 (11)
k_2	s^{-1}	26.6 ± 1.2 (11)	13.2 ± 1.1 (7)	25.6 ± 1.6 (11)
k_{-2}	s^{-1}	12.1 ± 1.3 (11)	23.0 ± 1.9 (7)	11.1 ± 1.5 (11)
K_2	—	2.64 ± 0.43 (11)	0.60 ± 0.08 (7)	3.47 ± 0.98 (11)
σ	—	0.72	0.37	0.77
k_4	s^{-1}	7.1 ± 0.6 (10)	11.4 ± 1.0 (13)	5.8 ± 0.3 (15)
k_{-4}	s^{-1}	12.6 ± 1.3 (10)	5.2 ± 0.6 (13)	11.1 ± 1.3 (15)
K_4	—	0.59 ± 0.04 (10)	2.58 ± 0.35 (13)	0.57 ± 0.04 (15)
K_5	mM^{-1}	0.14 ± 0.04 (10)	0.39 ± 0.08 (13)	0.18 ± 0.04 (15)

±SE. The number of observations is shown in parentheses.

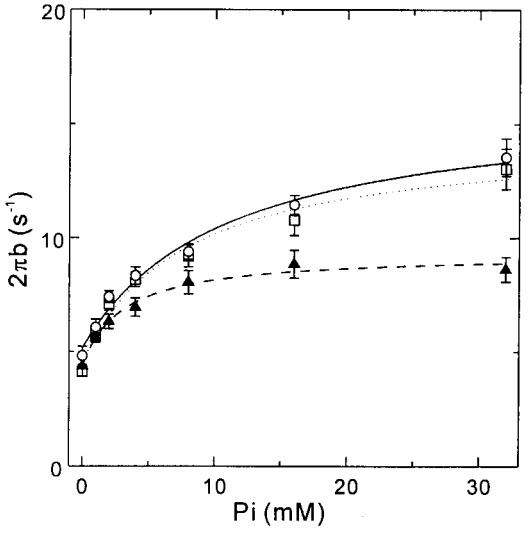


FIGURE 5 The rate constant $2\pi b$ is plotted as a function of Pi concentration. Bovine control myocardium ($n = 10$) (○ *and continuous line*); actin filament-reconstituted myocardium ($n = 13$) (▲ *and dashed line*); thin filament-reconstituted myocardium ($n = 15$) (□ *and dotted line*). Continuous curves are based on Eq. 3.

Isometric tension and stiffness

Fig. 7 shows isometric tension, stiffness, and isometric tension:stiffness ratio plotted against the MgATP concentration. In all muscle models, both isometric tension and stiffness decreased whereas tension/stiffness increased by the increase in the MgATP concentration. This result is in agreement with previous results in cardiac muscle fibers (Kawai et al., 1993) as well as in skeletal muscle fibers (Kawai and Zhao, 1993). In actin filament-reconstituted myocardium, isometric tension was less than control in all MgATP concentrations tested. In thin filament-reconstituted myocardium, isometric tension regained that of control myocardium. The tension:stiffness ratio also decreased in actin filament-reconstituted myocardium (0.76 × at 5 mM MgATP) but regained the original value in thin filament-reconstituted myocardium.

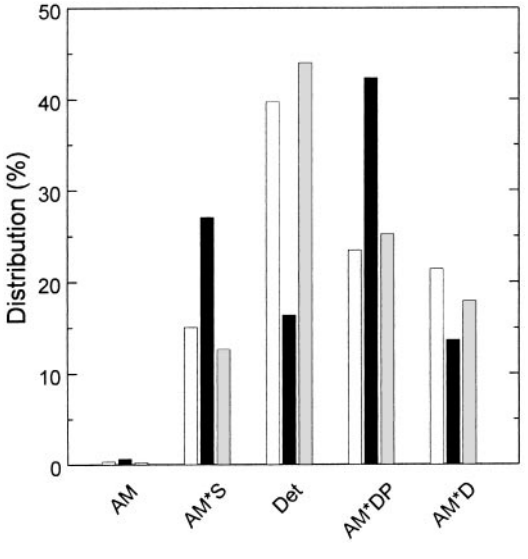


FIGURE 6 Calculated cross-bridge distribution in control (*white bars*), actin filament-reconstituted (*black bars*), and thin filament-reconstituted myocardium (*stippled bars*).

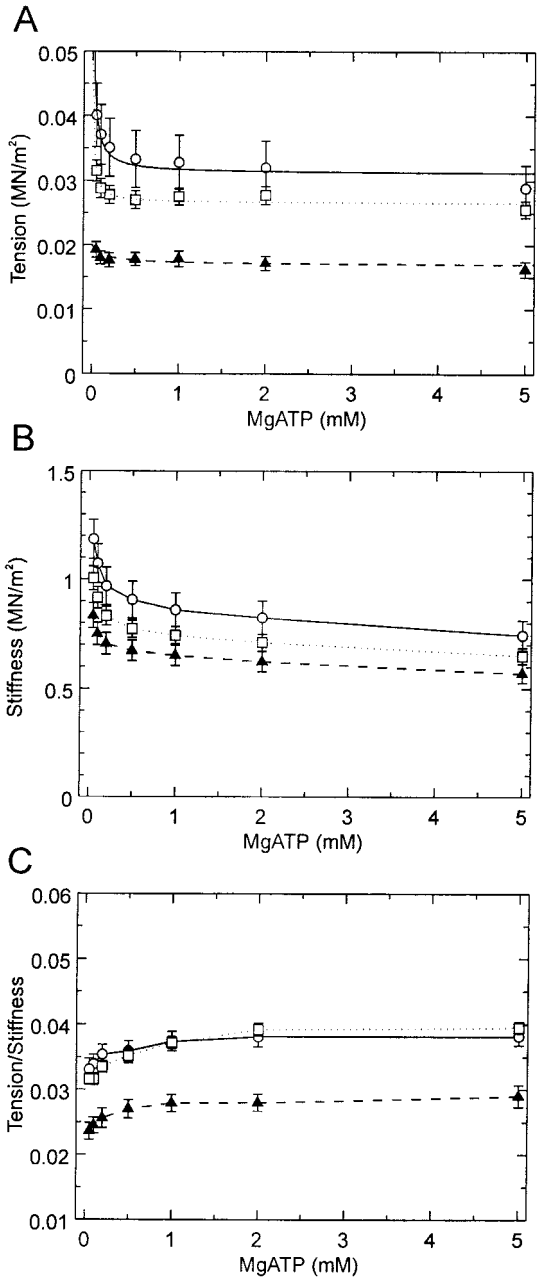


FIGURE 7 Isometric tension (*A*), stiffness (*B*), and tension/stiffness (*C*) are plotted against the MgATP concentration. Control myocardium ($n = 11$) (○ *and continuous line*); actin filament-reconstituted myocardium ($n = 13$) (▲ *and dashed line*); thin filament-reconstituted myocardium ($n = 11$) (□ *and dotted line*). Continuous lines in *A* are calculated based on Eq. 5 of Kawai and Zhao, 1993. Experiments were performed in the presence of 8 mM Pi.

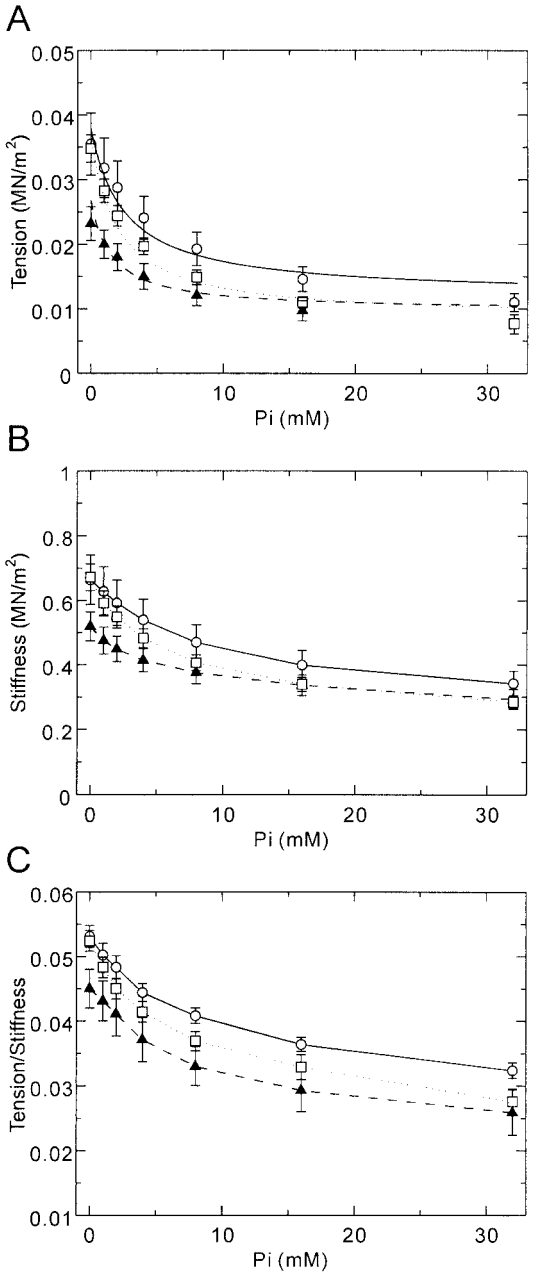


FIGURE 8 Isometric tension (*A*), stiffness (*B*), and tension/stiffness (*C*) are plotted against the Pi concentration. The same plotting nomenclatures were used as Fig. 7. Experiments were performed in the presence of 5 mM MgATP.

Fig. 8 shows isometric tension, stiffness, and the tension: stiffness ratio plotted against Pi concentration for the same experiments as shown in Fig. 5. In all muscle models, both isometric tension and stiffness decreased with the increase in the Pi concentration, which is in agreement with previous results in cardiac muscle fibers (Nosek et al., 1990; Kawai et al., 1993) and skeletal muscle fibers (Dantzig et al., 1992; Kawai and Zhao, 1993).

In thin filament-reconstituted myocardium, isometric tension regained that of control myocardium. The tension: stiffness ratio also decreased in actin filament-reconstituted myocardium (0.85× at no added Pi) but regained the original value in thin filament-reconstituted myocardium.

Isometric tension of accumulated data for MgATP and Pi studies at the standard activating condition (5 mM MgATP, 8 mM Pi) was $T_c = 24.3 \pm 2.4$ kN/m^2 ($n = 21$) in control myocardium. T_c was used for the normalizing factor in rigor stiffness. This tension value is in agreement with the previous results on ferret myocardium (Saeki et al., 1991), rat myocardium (Wannenburg et al., 2000), rabbit myocardium (Nosek et al., 1990), and porcine myocardium (Zhao and Kawai, 1996). The tension:stiffness ratio of accumulated data for MgATP and Pi studies at the standard condition was 0.039 ± 0.001 ($n = 21$) in control myocardium, 0.031 ± 0.002 ($n = 26$) in actin filament-reconstituted myocardium, and 0.038 ± 0.001 ($n = 26$) in thin filament-reconstituted myocardium.

Adequacy of the ATP regenerating system

Although it can be argued that the CK concentration that we used (320 U/ml) would be adequate to buffer against ADP build up, which is known to slow down the cross-bridge kinetics (Cooke and Pate, 1985; Kawai and Halvorson, 1991; Thirlwell et al., 1994; Martin and Barsotti, 1994), it would be better to demonstrate this point experimentally. We chose 0.1 mM MgATP concentration for this purpose, because both $2\pi b$ and $2\pi c$ are sensitive to the change in MgATP at this concentration (Fig. 4). We then increased the CK concentration and the CP concentration by 50% each (ionic strength was maintained at 200 mM) and the rate constants were compared. After the increase, the ratio (after: before) for $2\pi b$ was 0.99 ± 0.06, and for $2\pi c$ was 1.01 ± 0.03 ($n = 18$). As these numbers indicate, there is hardly any change in the rate constants when CK and CP concentrations were increased, demonstrating that MgATP concentration was adequately buffered, and that the ADP concentration was kept minimal so as not to affect our results.

Rigor stiffness

It is possible that the decreased ability of force generation in the actin filament-reconstituted myocardium may have been caused by an increased compliance (= 1/stiffness) of the actin filament, because of the absence of Tm and Tn in this preparation. To test this possibility directly, the myocardium was brought into the rigor condition (such as shown in Fig. 2 *D*), and its stiffness at 100 Hz was compared in three muscle models. The rigor stiffness is approximately a constant of frequency (Kawai and Brandt, 1980), hence the choice of this frequency does not alter the results. We found that rigor stiffness was 50 ± 3 T_c ($n = 21$) for control, $61 \pm$

4 T_c ($n = 26$) for the actin filament-reconstituted myocardium, and 52 ± 3 T_c ($n = 26$) for the thin filament-reconstituted myocardium. These results were normalized to the initial control tension (T_c) to avoid errors because of the cross-sectional area estimation. Thus, we found that the rigor stiffness was 1.2× larger in the absence of the regulatory system than in its presence, although the effect is small. The rigor stiffness may depend on several factors: stiffness of the actin filament, presence/absence of regulatory proteins, presence/absence of Ca^{2+} when regulatory proteins are present, actomyosin interface, stiffness of the myosin head, and stiffness of the thick filament. With our present experiments, it is not possible to determine which one of these factors contributes to a small change observed in the rigor stiffness.

DISCUSSION

Reconstitution of the actin filament with/without the regulatory system

We have succeeded in selectively removing the thin filament from bovine myocardium using plasma protein gelsolin purified from bovine serum. We also have succeeded in reconstituting the thin filament first with G-actin, then with Tm and Tn. In the present study, these results are confirmed by SDS-PAGE, isometric tension, and the rate and association constants of the elementary steps of the cross-bridge cycle. In previous studies (Fujita et al., 1996; Fujita and Ishiwata, 1998, 1999; Ishiwata et al., 1998), the reconstitution was confirmed by SDS-PAGE, electron microscopy, fluorescence microscopy, and isometric tension. In the current report, the reproducibility of isometric tension averaged 107 ± 4% after thin filament reconstitution. This good reproducibility of the isometric tension was achieved by 1) good temperature regulation during actin filament reconstitution; 2) shorter duration of reconstitution of the actin filament; and 3) choosing similar diameter of the myocardium. Reproducibility of isometric tension varied more in an earlier report (Fujita et al., 1996), but reproducibility has been improved in our later reports (Fujita and Ishiwata, 1998, 1999; Ishiwata et al., 1998), because these factors were more tightly controlled.

The fact that the rate constants of elementary steps in the control myocardium and the thin filament-reconstituted myocardium are the same within an experimental error (Table 2) implies that the myocardium is not damaged by the extraction, and that functional reconstitution is complete. Therefore, we are at a stage to be able to compare the significance of the regulatory system (Tm and Tn) on the elementary steps of the cross-bridge cycle. It has been generally assumed that the addition of the regulatory system enhances the contractile function, based on the fact that the ATP hydrolysis rate increased in solution (Bremel and Weber, 1972) and both gliding speed and force increased on in vitro motility assay (Gordon et al., 1998; Van Buren et al., 1999; Bing et al., 2000a, b; Homsher et al., 2000). This report is the first to demonstrate the significance of the regulatory system of the thin filament in a skinned fiber system under the physiological ionic strength of 200 mM. Previous reports were based on a low ionic strength (~50 mM) where ionic interaction is relatively more significant than hydrophobic interaction. Because force generation is primarily based on hydrophobic interaction (Kodama, 1985; Rayment et al., 1993; Zhao and Kawai, 1994), we believe that it is important to observe these results at physiological ionic strength. In previous attempts on muscle fibers, TnC can be removed and reconstituted without disturbing the contractile function (Brandt et al., 1990; Moss, 1992; Zhao et al., 1996), but there have been difficulties in removing and replacing TnT or TnI (Strauss et al., 1992). Because Tm is a rod-like protein, its selective removal has been difficult despite several attempts (Yanagida and Oosawa, 1975). In the current method, these components can be removed completely and reconstituted with those from purified proteins (Fig. 1).

Comparison of the kinetic constants in the presence and absence of the regulatory proteins

The most significant finding in the current study is that the presence of the regulatory system promotes cross-bridge detachment by increasing K_2 and decreasing K_4 (Table 2). The effect is four- to sixfold and substantial. K_5 (Pi association constant) decreases to promote cross-bridge attachment in the presence of the regulatory system, but its effect (2×) is not as large. Thus, these findings imply that the addition of the regulatory system inhibits actomyosin interaction even in the presence of Ca^{2+}. It is not difficult to visualize this inhibitory effect, because the primary role of the regulatory system is to be placed between actin and myosin and to inhibit their interaction. The interaction may not be fully removed even if Ca^{2+} is introduced in the myofilament space and binds to TnC. The fact that the regulatory proteins modify the rate and association constants of the elementary steps demonstrates that cross-bridge kinetics are under the influence of the regulatory proteins, as shown previously with TnC (Zhao et al., 1996).

Comparison of the kinetic constants among mammalian cardiac muscles

We have analyzed the contractile function of ferret (Kawai et al., 1993), porcine (Zhao and Kawai, 1996), and bovine myocardium in terms of the elementary steps of the cross-bridge cycle. These results demonstrate that the equilibrium constant of the force generation step (K_4) is smallest in ferret myocardium (0.11), intermediate in porcine (0.32), and largest in bovine myocardium (0.59). The results also

demonstrate that the Pi-association constant (K_5) is smallest in ferret myocardium (0.06 mM^{-1}), intermediate in porcine (0.10 mM^{-1}), and largest in bovine myocardium (0.14 mM^{-1}). Thus, it can be concluded that the strongly attached AM*DP state is most significantly populated in bovine myocardium, and least significantly populated in ferret myocardium under the same ionic conditions. This trend is also evidenced in the tension versus Pi plot in which a tension decrease with an increase in the Pi concentration is least in ferret myocardium, followed by bovine and porcine myocardium. Therefore, resistance toward Pi accumulation is best in ferret, followed by bovine and porcine. This conclusion may be related to the fact that small animals readily accumulate Pi because of higher metabolic need, as shown by the higher heart rate. Conversely, the heart rate of large animals is slow, hence the metabolic need can be less stringent, and the Pi accumulation may not be significant. These differences are presumably related to a sequence difference of myosin HC as well as that of light chains among various mammals.

With respect to the MgATP binding step 1 and subsequent dissociation step 2, there is an order of magnitude agreement of the results among skinned myocardium data and solution biochemistry data on isolated myosin subfragment 1 (S1). The MgATP binding step (K_1) of this report (9×10^3 M^{-1}, Table 2) compares with 5×10^3 M^{-1} on bovine cardiac S1 (Taylor and Weeds, 1976). Taylor and Weeds measured fluorescence enhancement in the absence of actin. The corresponding value for porcine myocardium is 11×10^3 M^{-1} (Zhao and Kawai, 1996), and for ferret myocardium 1.0×10^3 M^{-1} (Kawai et al., 1993). The cross-bridge detachment step (k_2) of this report (27 s^{-1}) compares with 13 s^{-1} for porcine myocardium (Zhao and Kawai, 1996), and 48 s^{-1} for ferret myocardium (Kawai et al., 1993). The corresponding value for bovine cardiac S1 is 100 s^{-1} for the isomerization step that follows MgATP binding (Taylor and Weeds, 1976). Once again, there is a good agreement among the results of the solution study and those of skinned myocardium. The above results indicate that MgATP binds ~10× stronger in myocardium in large animals (porcine, bovine) than in a small animal (ferret).

Other investigators observed steps 1 and 2 by using NPE-caged ATP on guinea pig trabeculae muscles (Martin and Barsotti, 1994). Although this caged ATP has a slow photolysis reaction (~100 s^{-1}), this rate does not limit the steps of the actomyosin cycle in myocardium, because myocardial cross-bridge reactions (Table 2) are generally slower than the photolysis reaction of caged ATP. Martin and Barsotti reported that the second-order rate constant of MgATP binding was 0.084×10^6 $M^{-1}s^{-1}$ in the presence of Ca^{2+} and when rigor preparations were treated with apyrase to remove residual MgADP. Our corresponding value (K_1k_2) for the ferret is 0.05×10^6 $M^{-1}s^{-1}$ (Kawai et al., 1993); for the porcine is 0.14×10^6 $M^{-1}s^{-1}$ (Zhao and Kawai, 1996); and for the bovine is 0.11×10^6 $M^{-1}s^{-1}$ (Table 2). The value obtained from S1 of bovine cardiac muscle is 0.5×10^6 $M^{-1}s^{-1}$ (Taylor and Weeds, 1976). Again, a general agreement can be seen among the measurements. This agreement is striking when we realize that very different techniques and conditions were used in these measurements. The method that used caged ATP was not able to separate steps 1 and 2, presumably because the ATP concentration released by photolysis of caged ATP was limited to 2 mM (Martin and Barsotti, 1994), hence saturation of the rate constant could not have been observed. With our experience using ferret myocardium (which may be comparable with guinea pig myocardium), saturation of $2\pi c$ is barely visible at 2.5 mM but apparent at 5 mM MgATP (Kawai et al., 1993). This saturation is essential to characterize the ATP isomerization/cross-bridge detachment step.

The slowest step of the cross-bridge cycle

Step 6 is a transition from the AM*D state to AMD and/or to the AM state, and the slowest forward step in the cross-bridge cycle. The ATP hydrolysis rate is determined by k_6[AM*D] (Kawai and Zhao, 1993). Our result that the probability of the AM*D state being larger in the presence of Tm and Tn (Fig. 6) implies that the ATP hydrolysis rate would be larger if the actin filament is reconstituted with these regulatory proteins. This mechanism is consistent with an earlier observation (Bremel and Weber, 1972) that the ATP hydrolysis rate is larger in the presence of Tm and Tn in solution.

The rate constant of step 6 (k_6) may be relatively more significant in cardiac muscles than in skeletal muscles. This causes cross-bridges distributed in AM through AM*DP states even in the absence of added Pi. Our finding that the endogenous Pi concentration (P_0 ~1.1 mM) is higher than that of rabbit psoas fibers (0.6 mM; Kawai and Halvorson, 1991) implies that cross-bridges are indeed distributed in AM through AM*DP states in the absence of added phosphate, which is consistent with the hypothesis that the role of k_6 is more significant in cardiac muscles than in fast-twitch skeletal muscles.

Force supported by each cross-bridge

Fig. 6 demonstrates that, in the control and thin filament-reconstituted myocardium, ~44% of active cross-bridges are in the AM*DP and AM*D states that generate force. In contrast, in the actin filament-reconstituted myocardium, 56% of cross-bridges are attached in these states. Thus, ~1.3× (= 56/44) more cross-bridges are formed in the force generating states in the absence of the regulatory system. Fig. 2 *C* demonstrates that force is smaller and 0.7× compared with the control (Fig. 2 *A*). From these data, it can be concluded that force supported by strongly attached cross-bridges is 0.5× (= 0.7/1.3) in the absence of the

regulatory system. This argument is based on the hypothesis that the Pi release consists of steps 4 and 5, and that force is generated on step 4 (isomerization) and the same force is maintained after actual Pi release (step 5) based on lines of experimental evidence (Fortune et al., 1991; Kawai and Halvorson, 1991; Dantzig et al., 1992).

Although this hypothesis may appear in contradiction to a generally accepted assumption that force generation occurs with Pi release, it is not. This is because the general assumption was based on solution studies that did not recognize the strongly attached AM*DP state, but recognized that the Pi-release step was highly unidirectional, requiring 10–100 M Pi to reverse the equilibrium (Taylor, 1979). Because such a reaction accompanies a large, free-energy reduction, it has been generally assumed that this energy is used for force generation. In the muscle fiber system, it has been known that the Pi-release step is reversible with the millimolar Pi concentration (Bowater and Sleep, 1988; Kawai and Halvorson, 1991). In the fiber system, both a weakly attached AMDP state and a strongly attached AM*DP state have been recognized (Fortune et al., 1991; Kawai and Halvorson, 1991; Dantzig et al., 1992).

The above argument assumes that only the AM*DP and AM*D states are strongly attached force-generating states, but the argument is not much different if we include the AM and AMS states, because the cross-bridge distribution in the AM state is very small ($\leq$ 0.6%) (Fig. 6), and force supported by the AMS state is perhaps half of that supported by the AM*DP or AM*D state (Kawai and Zhao, 1993). Our observations, that in the absence of the regulatory system the tension:stiffness ratio is smaller by $\sim$0.8$\times$, and rigor stiffness is larger by 1.2$\times$, also imply that the tension supported by each cross-bridge is smaller than in the presence of the regulatory system by $\sim$0.7$\times$ (= 0.8/1.2). Although the two estimates (0.5$\times$ vs. 0.7$\times$) do not match exactly, presumably because of errors associated with each estimate, the conclusion that the force supported by each cross-bridge is less in the absence of the regulatory system seems to be solid. The decrease in force-generating capacity in the actin filament-reconstituted myocardium is not attributable to an increased compliance of the filament, because our stiffness measurement during rigor induction demonstrates that the stiffness of actin filament-reconstituted myocardium is no less than that of the control or the thin filament-reconstituted myocardium. Our result on myocardium is consistent with recent results that used the in vitro motility assay, demonstrating that the gliding speed increased by 1.4–1.8$\times$ when Tm and Tn were added (Gordon et al., 1998; Bing et al., 2000a; Homsher et al., 2000), and demonstrating that force also increased by 1.5–3.0$\times$ when Tm and Tn were added (Homsher et al., 2000; Bing et al., 2000b). Geeves and Halsall (1986) showed actomyosin-binding affinity was increased in the presence of Tm and Tn in solution.

It is interesting to ask why force depends on the regulatory system. After the critical power stroke that takes place in step 4, the amount of force development must be a result of macromolecular architecture between actin and myosin molecules. If Tm is present together with Tn and Ca^{2+}, these molecules may either alter the conformation of actin, or intervene between actin and myosin to modify their relationship. The latter mechanism is less likely as a direct interaction between myosin and Tm has not been demonstrated, except that they are known to come close together. If the conformation of actin is altered by Tm, it is almost certain that this alteration affects the actin-myosin interaction surface, hence the amount of force generation.

A line of evidence suggests that Tm plays a role in the cross-bridge kinetics that result in force generation. Fujita and Ishiwata (1999) showed that the pH effect of isometric tension depends on a Tm isoform, demonstrating the significance of Tm in force generation. When Tm was added to the in vitro motility assay system, Van Buren at al. (1999) reported a 2$\times$ increase in force measured between single actin filament and myosin molecules coated on a glass surface, and Bing et al. (2000a) reported a 1.20–1.25$\times$ increase in sliding velocity. Another line of evidence suggests that the Tn complex plays a role in the cross-bridge cycling and concomitant force generation. It was shown that the phosphorylation of TnI increased relaxation and decreased Ca^{2+} sensitivity in the rabbit heart (Solaro et al., 1976; Zhang et al., 1995; Solaro and Van Eyk, 1996; Kajiwara et al., 2000). Sweeney et al. (1998) and Homsher et al. (2000) demonstrated that TnT mutant affected sliding speed in an in vitro motility assay. Zhao et al. (1996) showed that TnC removal resulted in a decrease in the rate constant of the force-generation step in rabbit psoas fibers.

The current study demonstrates that force per cross-bridge is enhanced by 1.5$\sim$1.7$\times$ in the presence of Tm and Tn in myocardium. These results imply that the signal transmission through TnC, TnI, TnT, Tm, actin, and myosin results in a conformational change at the interface of actin and myosin. A partial model consistent to this hypothesis was proposed by Tobacman and Butters (2000) based on actomyosin interaction in solution using internal deletion mutants of Tm, in which they showed that the interaction increased by sevenfold when Tm was present. The technique developed in this study would be useful to elucidate the role of molecular domains of contractile proteins by using mutants of thin filament proteins.

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