

Cross-Bridge versus Thin Filament Contributions to the Level and Rate of Force Development in Cardiac Muscle

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ABSTRACT In striated muscle thin filament activation is initiated by Ca^{2+} binding to troponin C and augmented by strong myosin binding to actin (cross-bridge formation). Several lines of evidence have led us to hypothesize that thin filament properties may limit the level and rate of force development in cardiac muscle at all levels of Ca^{2+} activation. As a test of this hypothesis we varied the cross-bridge contribution to thin filament activation by substituting 2 deoxy-ATP (dATP; a strong cross-bridge augments) for ATP as the contractile substrate and compared steady-state force and stiffness, and the rate of force redevelopment (k_{tr}) in demembranated rat cardiac trabeculae as $[\text{Ca}^{2+}]$ was varied. We also tested whether thin filament dynamics limits force development kinetics during maximal Ca^{2+} activation by comparing the rate of force development (k_{Ca}) after a step increase in $[\text{Ca}^{2+}]$ with photorelease of Ca^{2+} from NP-EGTA to maximal k_{tr} , where Ca^{2+} binding to thin filaments should be in (near) equilibrium during force redevelopment. dATP enhanced steady-state force and stiffness at all levels of Ca^{2+} activation. At similar submaximal levels of steady-state force there was no increase in k_{tr} with dATP, but k_{tr} was enhanced at higher Ca^{2+} concentrations, resulting in an extension (not elevation) of the k_{tr} -force relationship. Interestingly, we found that maximal k_{tr} was faster than k_{Ca} , and that dATP increased both by a similar amount. Our data suggest the dynamics of Ca^{2+} -mediated thin filament activation limits the rate that force develops in rat cardiac muscle, even at saturating levels of Ca^{2+} .

INTRODUCTION

Striated muscle contraction results from cyclic interactions between myosin and actin, which are regulated by the troponin-tropomyosin complex of the thin filament. Ca^{2+} binding to the thin filament regulatory protein troponin C (TnC) initiates contraction (Gordon et al., 2000) and the level of force developed is determined by the availability of myosin binding sites on thin filaments, which is ultimately controlled by the distribution of tropomyosin (Tm) between positions on the surface of thin filaments that either block or uncover binding sites on actin for myosin cross-bridge attachment. Transduction of the Ca^{2+} signal is through the troponin complex, resulting in increased mobility of Tm over the surface of actin and exposure of strong binding sites for myosin. This shifts the thin filament from a “blocked” to a “closed” state (McKillop and Geeves, 1993), thereby allowing further myosin cross-bridge formation. Strong cross-bridge binding is then thought to stabilize Tm in an “open” state and the thin filament is considered “activated.” In effect, the level of contractile activation appears to result from a balance of the effects of Ca^{2+} binding to TnC and cross-bridges on Tm state. The growing amount of evidence suggests that cross-bridge displacement of Tm into an activating position may play a more prominent role in the regulation of cardiac contraction (Fuchs and Smith, 2001).

Like force, the rate of force development is Ca^{2+} dependent, but the mechanisms that determine the thin filament activation dependence of force kinetics are less well established. In demembranated skeletal muscle fibers the relationship between steady-state force and the rate that force redevelops (k_{tr}) after a release-restretch cycle is Ca^{2+} sensitive, being slow ($1\text{--}2\text{ s}^{-1}$ at $10\text{--}15^\circ\text{C}$) at low levels of force and increasing 10–15-fold as force rises to maximum at high levels of Ca^{2+} activation (Brenner, 1986, 1988). Based on these and other findings it was suggested that Ca^{2+} controls the rate-limiting process in force development rather than simply controlling the level of cross-bridge recruitment (Brenner, 1986, 1988). However, we and others have shown that in skeletal muscle when the Ca^{2+} -activated force is high the kinetics of actin-myosin interaction are likely limiting to the rate of force development, whereas at submaximal force the rate-limiting process may be the kinetics of thin filament activation (Chase et al., 1994; Regnier et al., 1996, 1998; Fitzsimmons et al., 2001a,b). This limiting process is greatest at low levels of activation, as most of the increase in k_{tr} comes at Ca^{2+} concentrations that produce $>50\%$ of maximal Ca^{2+} -activated steady-state force.

Similar to reports for fast skeletal muscle, k_{tr} is Ca^{2+} dependent in demembranated cardiac muscle. However, most studies have reported smaller magnitude (one- to fivefold) change in k_{tr} (compared with skeletal muscle) as the level of Ca^{2+} activation is varied (Wolff et al., 1995; Palmer and Kentish, 1998; Patel et al., 2001), though one recent report suggest a >8 -fold change (Fitzsimmons et al., 2001). These studies mostly show that at similar temperatures k_{tr} is similar or faster in cardiac versus skeletal muscle at low Ca^{2+}

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concentrations, and slower at high levels of Ca^{2+} activation. This suggests the lower Ca^{2+} dependence of k_{tr} results from different actin-myosin cycling kinetics (the main determinant of maximal k_{tr} in skeletal muscle) and/or the relative kinetics of thin filament processes versus actin-myosin cycling kinetics is different than in skeletal muscle (Hancock et al., 1997). Coupled with evidence that thin filament activation level is more dependent on strong cross-bridge binding in cardiac (versus skeletal) muscle, it also suggests that Ca^{2+} binding to Tn may result in less complete activation of cardiac thin filaments (Regnier et al., 2000b).

We have been studying the relative contribution of Ca^{2+} binding to TnC and cross-bridge binding on both the level of thin filament activation and the rate that force develops in cardiac and skeletal muscle. A particularly successful approach has been to alter cross-bridge binding and cycling rates by using nucleotide analogs of ATP (NTPs) as the substrate for contraction. We have shown that one of these analogs, 2 deoxy-ATP (dATP), has an affinity for skeletal myosin similar to that of ATP and increases both solution NTPase and unloaded shortening velocity of rabbit skeletal and rat cardiac muscle (Regnier and Homsher, 1998; Regnier et al., 1998, 2000b). Interestingly, dATP enhances both maximal Ca^{2+} -activated steady-state force (F_{max}) and k_{tr} in cardiac trabeculae from both untreated rats, containing mostly α -MHC (fast cardiac myosin), and PTU-treated rats, containing β -MHC (slow cardiac myosin) (Regnier et al., 2000b). This contrasts with our findings in skeletal muscle where dATP has no effect on F_{max} , but increases maximal k_{tr} in rabbit psoas fibers (fast skeletal myosin) and rat soleus fibers (slow skeletal myosin) (Regnier et al., 1998). These results indicate that cross-bridge cycling rate per se cannot explain these cardiac versus skeletal muscle differences, and suggests that the properties of cardiac thin filament regulatory proteins may limit cross-bridge cycling. Therefore we have hypothesized that (compared to ATP) dATP augments the level of thin filament activation and/or strong cross-bridge binding at saturating Ca^{2+} concentration in demembranated cardiac muscle, resulting in an increase in F_{max} and maximal k_{tr} over that attained with ATP as the contractile substrate. This hypothesis is supported by our finding that dATP also enhances both force and k_{tr} at subsaturating levels of Ca^{2+} activation in skeletal muscle (Regnier et al., 1998).

In this study we hypothesized that the relatively low activation dependence of k_{tr} in cardiac muscle (compared with skeletal muscle) reflects a relative limitation of Ca^{2+} binding to TnC to activate thin filaments at all levels of Ca^{2+} . Consequently, this would give cross-bridge binding a more prominent role in the activation process. To test this hypothesis we compared steady-state force and stiffness, and k_{tr} in rat cardiac trabeculae as Ca^{2+} concentration was varied with either 5 mM ATP or 5 mM dATP as the contractile substrate. In addition, we tested the hypothesis that thin filament kinetics can limit the rate of force development during maximal Ca^{2+} activation. To do this we compared the

rate force develops after a step increase in Ca^{2+} concentration with photorelease of Ca^{2+} from NP-EGTA (k_{Ca}) (Ellis-Davies and Kaplan, 1994) to maximal k_{tr} , where Ca^{2+} binding to thin filaments should be in (near) equilibrium during force development. We found that dATP enhanced steady-state force and stiffness at all levels of Ca^{2+} activation. We also found that at similar submaximal levels of steady-state force there was no increase in k_{tr} with dATP, but dATP enhanced k_{tr} at higher Ca^{2+} concentrations, resulting in an extension (not elevation) of the curvilinear force- k_{tr} relationship. Similar results were obtained for rat trabeculae with either α -MHC or β -MHC isoforms, indicating this effect was not due to the absolute rate of cross-bridge cycling. Significantly, we found that k_{tr} is faster than k_{Ca} with maximal Ca^{2+} activation, and that both are increased a similar amount by dATP. This finding suggests the kinetics and/or level of Ca^{2+} -mediated thin filament activation may limit the rate that force develops in rat cardiac muscle, even at saturating levels of Ca^{2+} . In a subsequent article, (Adhikari et al., 2004), we show that this property of cardiac thin filaments may at least partially explain the greater sarcomere length-dependence of force found in cardiac (versus skeletal) muscle. Preliminary reports of this work have been published previously (Regnier et al., 2000a; Martin et al., 2002).

METHODS

Male Sprague-Dawley rats (200–250 grams), either treated with propylthiouracil (PTU; 0.5% added to drinking water for 4–8 weeks) or untreated (as age-matched animals) were euthanized with sodium pentobarbital (50 mg/kg). Hearts were excised and the interior wall of the right ventricle exposed to relaxing solution (see below) containing glycerol (50% v/v) and Triton X-100 (1%) overnight at 5°C. Individual trabeculae were dissected and stored at 5°C for up to 4 days.

Solutions

Experimental solutions contained (in mM): 15 phosphocreatine, 15 EGTA, at least 40 3-(*N*-morpholino)propanesulfonic acid (MOPS), 1 free Mg^{2+} , 135 Na^+ + K^+ , 1 dithiothreitol (DTT), 250 units ml^{-1} creatine kinase (CK, Sigma, St. Louis, MO), and either 5 ATP or 5 mM 2 deoxy-ATP (Sigma, St. Louis, MO) at pH 7.0 and $15 \pm 1^\circ\text{C}$. Ionic strength was 0.2 M. Affinities of dATP and ATP for Mg^{2+} were assumed to be the same (Regnier and Homsher, 1998). For activation solutions, the Ca^{2+} level (expressed as $\text{pCa} = -\log [\text{Ca}^{2+}]$) was set by adjusting $\text{Ca}(\text{propionate})_2$.

Individual chemically demembranated ("skinned") trabeculae were wrapped in aluminum foil T-clips for attachment to a force transducer (either Model 400A, 2.2 kHz-resonant frequency (Cambridge Technology, Watertown, MA) or Model AE801, ≥ 5 kHz resonant frequency (SenoNor, Horton, Norway)) and a servo-motor (model 300, Cambridge Technology, Watertown, MA) tuned for a 300 μs -step response. For most experiments trabecular sarcomere length (L_s) was measured with helium-neon laser diffraction (Chase et al., 1993) and set to 2.25 μm at pCa 9.2. At this L_s trabeculae lengths ranged from 1.2–2.3 mm and diameters averaged 140 ± 16 μm , with no significant difference between trabeculae from normal versus PTU-treated rats. Stiffness was determined by small amplitude (0.05% L_s) sinusoidal length oscillations (1 kHz). Steady-state isometric force is reported as kN/M^2 in Results, assuming circular geometry and the rate of isometric tension redevelopment (k_{tr}) was determined from the

half-time of force recovery after a rapid release-restretch transient, as previously described (Regnier et al., 1998). For comparisons, force and k_{tr} are reported normalized to measurements obtained during maximal Ca^{2+} activation with ATP as the contractile substrate.

Comparisons of maximal k_{tr} with the rate of force development (k_{Ca}) after photorelease of Ca^{2+} from NP-EGTA were made during the same activation done in a separate set of experiments under identical conditions, except temperature was 17°C instead of 15°C . k_{Ca} was measured as described in Martin et al. (2004). Briefly, after initial Ca^{2+} activations in either ATP or dATP, during which control measurements of k_{tr} were performed at maximal activation ($k_{tr\text{ control}}$), the preparation was transferred through a series of NTP containing solutions to a final photolysis solution containing 5 mM NTP, 2mM NP-EGTA, and ~ 1.8 mM added calcium. The output from a frequency doubled ruby laser producing ~ 150 mJ in a 50 ns pulse at a wavelength of 347 nm (Laser Applications, Winter Park, FL) was focused onto the trabeculae. Incident energy was measured by deflecting 10% of the beam energy onto a Scientech Energy Meter (365, Boulder, CO); ~ 2 s after peak force was attained, k_{tr} was measured ($k_{tr\text{ photolysis}}$) and the tissue relaxed. For these experiments, all elicited transients were collected for subsequent analysis but only those resulting in maximal activations within 5% of maximal Ca^{2+} -activated force at pCa 4.0 (F_{max}) have been included in the data.

Data analysis

Force-pCa and k_{tr} -pCa relationships are fit with the Hill equation:

$$F = F_{\text{max}} / (1 + 10^{n_H(\text{pCa}_{50} - \text{pCa})}). \quad (1)$$

From each Hill fit, the pCa at half-maximal force or k_{tr} (pCa_{50}) and the slope (n_H) are determined, and reported pCa_{50} and n_H values represent the means of the values from the individual fits, \pm SE. The time course of force development from k_{tr} and k_{Ca} protocols was determined by exponential fits or from $t_{1/2}$ values, as previously described in (Chase et al., 1994), both yielding similar results. Summarized data are reported as mean \pm SE and means were compared using Student's t -test, with significance at the 95% confidence level.

RESULTS

Steady-state force

In chemically demembranated (skinned) trabeculae, substitution of 5 mM dATP for 5 mM ATP increased steady-state force at all levels of Ca^{2+} activation. The steady-state force data for all the experiments is summarized in Fig. 1. Trabeculae from untreated and PTU-treated rats were used to determine if force enhancement by dATP depends on the myosin isoforms present. Force values were normalized to F_{max} with ATP for each trabecula and the Ca^{2+} -dependence of force was characterized from fitting the Hill equation to the data (Eq. 1 in Methods) to obtain 1), the $[\text{Ca}^{2+}]$ required to elicit half of maximal Ca^{2+} -activated force (i.e., pCa_{50} or Ca^{2+} sensitivity); and 2), the slope (n_H or Hill coefficient) of the steep portion of the force-pCa relationship. For trabeculae from untreated rats F_{max} was 20.0 ± 4.2 kN/m² with ATP ($n = 8$) and was increased by $31 \pm 3\%$ with dATP (Fig. 1 *a*). In trabeculae from PTU-treated rats F_{max} was 17.6 ± 4.2 kN/m² with ATP ($n = 10$) and was increased by $39 \pm 4\%$ with dATP (Fig. 1 *b*). These increases in F_{max} with dATP are similar to what we have previously reported (Regnier

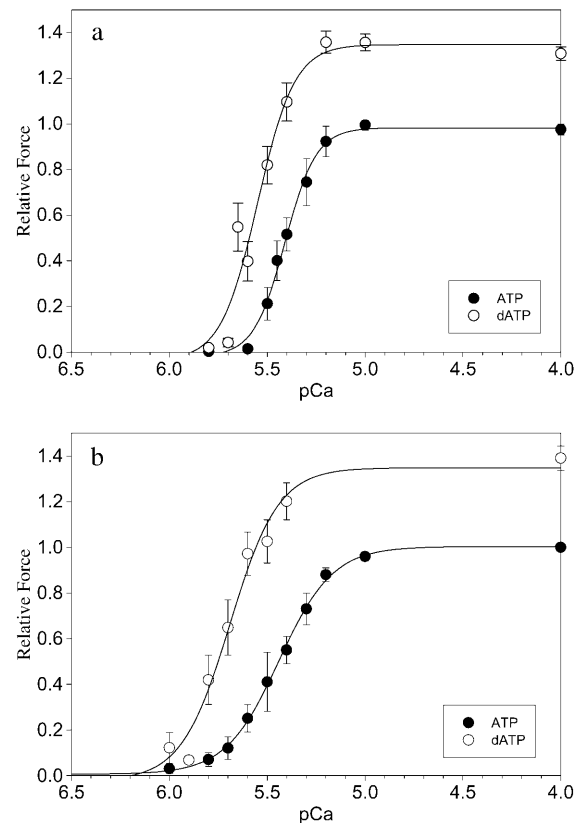


FIGURE 1 dATP (○) enhances the Ca^{2+} -sensitivity of steady-state force in skinned trabeculae. Force was normalized to maximal force at pCa 4.5 with ATP (●) for trabeculae from normal (*a*) and PTU-treated (*b*) rats for comparisons. Data are reported as the mean \pm SE for each pCa. Data were fit to Eq. 1 (Methods) and values for pCa_{50} and n_H are reported in the text of Results. Fits for the data were $r^2 > 0.997$.

et al., 2000b). dATP increased the Ca^{2+} -sensitivity of force (pCa_{50}) of trabeculae from both untreated and PTU-treated rats, but had no significant effect on n_H . For trabeculae from untreated rats, dATP increased the Ca^{2+} -sensitivity of force ($P < 0.05$) by 0.13 pCa units from 5.41 ± 0.01 (ATP) to 5.54 ± 0.03 (dATP), whereas corresponding n_H values were 5.4 ± 0.6 (ATP) and 4.6 ± 1.2 (dATP). For trabeculae from PTU-treated rats, pCa_{50} with ATP was similar to untreated rats, whereas the increase of Ca^{2+} sensitivity of force with dATP was greater than found for untreated rats, increasing 0.26 pCa units from 5.43 ± 0.01 (ATP) to 5.69 ± 0.03 (dATP). n_H was unaffected by dATP in PTU treated trabeculae (3.2 ± 0.2 (ATP) vs. 3.5 ± 0.6 (dATP)). The Hill fit parameters (pCa_{50} and n_H) for the force-pCa relations for trabeculae from untreated and PTU-treated rats included in Table 1.

In a subgroup of trabeculae from PTU-treated rats, steady-state stiffness was measured with small amplitude oscillations (see Methods). Linear regression analysis of the stiffness versus force relationship (as Ca^{2+} was varied) for activations in ATP versus dATP demonstrated a proportional increase in

TABLE 1 Comparison of Hill fit parameters for force-pCa and k_{tr} -pCa relations in untreated (control) and PTU-treated rats

	NTP	F_{max} (normalized)	pCa ₅₀ (force)	n_H (force)	Max. k_{tr} (normalized)	pCa ₅₀ (k_{tr})
Untreated	ATP (8)	1.0	5.41 ± 0.01	5.4 ± 0.6	1	5.28 ± 0.01
	dATP (8)	1.31 ± 0.03	5.54 ± 0.03	4.6 ± 1.2	1.27 ± 0.05	5.38 ± 0.01
PTU	ATP (10)	1.0	5.43 ± 0.01	3.2 ± 0.2	1	5.42 ± 0.01
	dATP (10)	1.39 ± 0.04	5.69 ± 0.03	3.5 ± 0.6	1.47 ± 0.04	5.47 ± 0.01

The number of trabeculae used for each NTP condition is given in parentheses. F_{max} and maximal k_{tr} values are normalized to force obtained with 5 mM ATP at pCa 4.5.

stiffness versus force for both nucleotides (Fig. 2). This suggests the increased force with dATP at each pCa results from enhanced strong cross-bridge binding.

Rate of force redevelopment (k_{tr})

Similar to the results with steady-state force, dATP enhanced k_{tr} at varying levels of Ca^{2+} activation. The force traces in Fig. 3 show this enhancement for example trabeculae from an untreated (Fig. 3 *a*) and a PTU-treated (Fig. 3 *b*) rat during activations at pCa 5.5 and pCa 4.0. In both of these examples trabeculae dATP elevated steady-state force at pCa 5.5 and pCa 4.0 by similar amounts versus ATP, and at pCa 5.5, k_{tr} was approximately doubled whereas maximal k_{tr} (pCa 4.0) was increased by 27 ± 5% (untreated) and 47 ± 4% (PTU-treated). Fig. 4 summarizes k_{tr} measurements at varying levels of activating Ca^{2+} , normalized to pCa 4.0 and ATP. At 15°C maximal k_{tr} with ATP was 10.6 ± 0.8 s⁻¹ (n = 8) for trabecula from untreated rats and 3.0 ± 0.2 s⁻¹ (n = 10) for trabeculae from PTU-treated rats. The data show that dATP increased k_{tr} at higher levels of Ca^{2+} activation, but had little or no effect on k_{tr} at the lowest levels of activation.

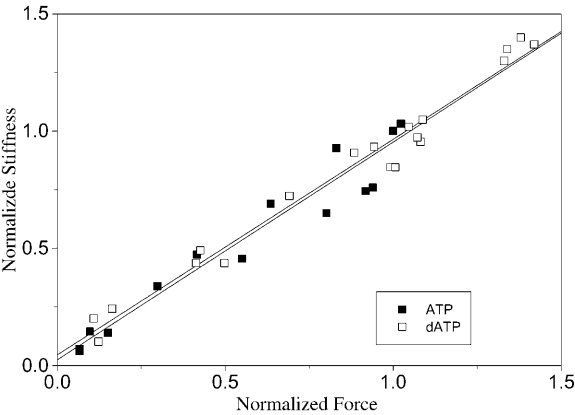


FIGURE 2 Relationship between steady-state force and trabeculae stiffness with varying $[Ca^{2+}]$ for 3 trabeculae from PTU-treated rats. Force and stiffness values are normalized to the level measured at pCa 4.0 with ATP as the contractile substrate. Linear regression fits to the ATP (■) and dATP (□) data yielded identical slopes of 0.96 with r^2 values >0.97. The values for dATP >1.0 demonstrate that trabeculae stiffness is elevated proportionally with force over values for ATP, suggesting an increase in the number of strongly bound, force-producing cross-bridges at high levels of Ca^{2+} activation.

Although the starting level of force after restretch of muscle fibers varied somewhat between preparations and between measurements with ATP versus dATP within the same preparation, there was no indication these variable starting-force levels affected the magnitude differences in k_{tr} between ATP and dATP. Hill fits to the data indicate an increase in the Ca^{2+} sensitivity (pCa₅₀) of k_{tr} with dATP. In trabeculae from untreated rats (Fig. 4 *a*) pCa₅₀ was increased by 0.10 units (P < 0.05) from 5.28 ± 0.01 (ATP) to 5.38 ± 0.01 (dATP). This increase in the Ca^{2+} sensitivity of k_{tr} was similar to the increase seen for steady-state force (Fig. 1 *a*) and stiffness (Fig. 2). For trabeculae from PTU-treated rats

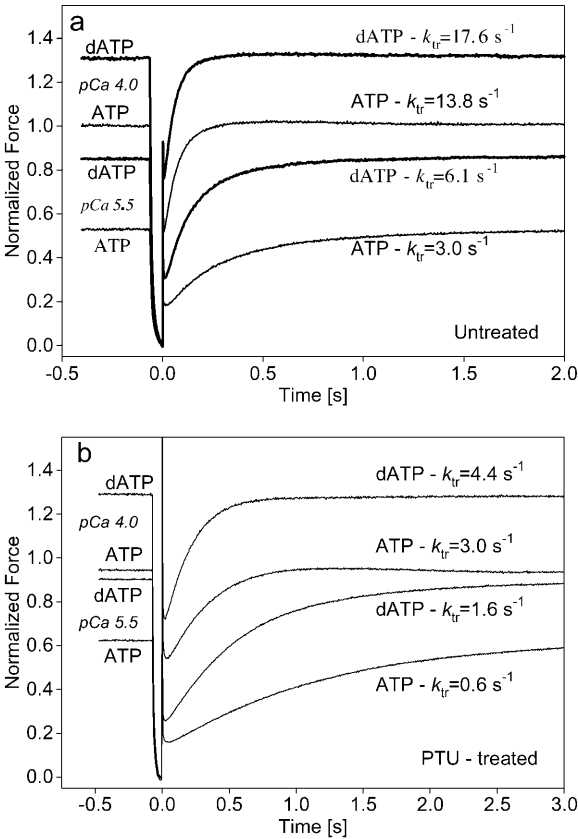


FIGURE 3 Example force records comparing k_{tr} with ATP versus dATP at pCa 5.5 and 4.0 for trabeculae from an untreated (*a*) and a PTU-treated (*b*) rat. The pCa and rate of force redevelopment (k_{tr}) for each trace are given next to the respective force records. These examples demonstrate elevation of both steady-state force and k_{tr} at submaximal and maximal levels of Ca^{2+} activation.

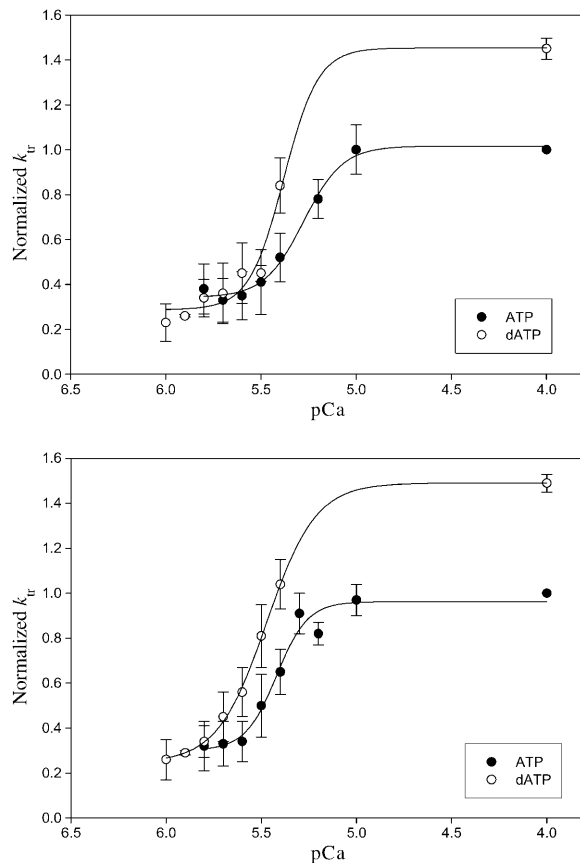


FIGURE 4 The Ca^{2+} dependence of k_{tr} with ATP (●) versus dATP (○) for trabeculae from untreated (a) and PTU-treated (b) rats. Measurements with ATP and dATP are made in the same preparations and values are normalized to maximal k_{tr} at pCa 4.0 with ATP. Values are mean \pm SE. Data were fit with Eq. 1 (Methods) to demonstrate an increase in the Ca^{2+} sensitivity (pCa_{50}) of k_{tr} , as well as maximal k_{tr} , with dATP in both (a) and (b).

(Fig. 4 b), the pCa_{50} of k_{tr} was increased by 0.05 units ($P < 0.05$) from 5.42 ± 0.01 (ATP) to 5.47 ± 0.01 (dATP), an increase not as profound as that seen for steady-state force (Fig. 1 b). These data are included in Table 1 for comparison to the corresponding steady-state force values.

The leftward shift observed for both the force versus pCa (Fig. 1) and k_{tr} versus pCa (Fig. 4) relationships suggest that increases in k_{tr} with dATP may have resulted from an increased number of cross-bridges available to participate in the force-generating process. To test this idea, the k_{tr} data in Fig. 4 were replotted as k_{tr} versus force (both normalized to pCa 4.0 with ATP) at each level of Ca^{2+} in activation solutions (Fig. 5). This replotting of the data clearly demonstrates that k_{tr} was similar for dATP during Ca^{2+} -activated contractions producing similar levels of steady-state force and was elevated by dATP only at activation levels resulting in greater steady-state force than that obtained with ATP during maximal Ca^{2+} activation. When coupled with the stiffness data, which rises proportionally with force (Fig. 2), it also suggests that k_{tr} with dATP is increased because of a greater

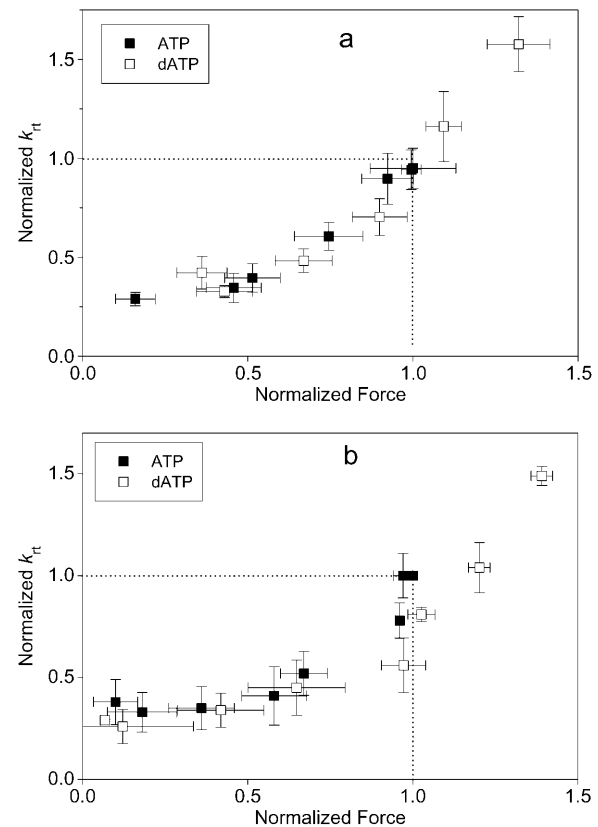


FIGURE 5 The relationship between steady-state force and k_{tr} as pCa is varied with ATP (■) versus dATP (□) for trabeculae from untreated (a) and PTU-treated (b) rats. Values are mean \pm SE. In both a and b, k_{tr} is not different for ATP versus dATP at similar levels of steady-state force, but dATP extends the curve beyond maximal values of force and k_{tr} for ATP (demonstrated by dotted lines) at high levels of Ca^{2+} activation.

recruitment of force generating cross-bridges at each level of activating Ca^{2+} , including during maximal Ca^{2+} activation.

Rate of force development (k_{Ca})

To investigate how Ca^{2+} activation processes might limit the rate of force development in cardiac muscle we compared the rate of force development under conditions where the $[\text{Ca}^{2+}]$ is kept constant (k_{tr}) versus when activation is initiated by a step increase in $[\text{Ca}^{2+}]$ by laser photolysis of NP-EGTA (k_{Ca}). Fig. 6 (upper panel) shows an example of the protocol used for these comparisons. This trabecula was placed in a control activation solution (pCa 4.5, 5 mM ATP) to determine F_{max} and maximal k_{tr} (first arrow), then was placed in relaxing solution (pCa 8.0). Next the trabecula was transferred into the NP-EGTA activation solution to allow the caged- Ca^{2+} to diffuse into the contractile lattice (2–3 min.). At this point the trabecula was activated by photolysis of the NP-EGTA (second arrow) and steady-state force developed, ~ 2 s later a k_{tr} measurement was made (third arrow) and the trabecula was relaxed. In several preparations the k_{tr} measurement was repeated in control activation

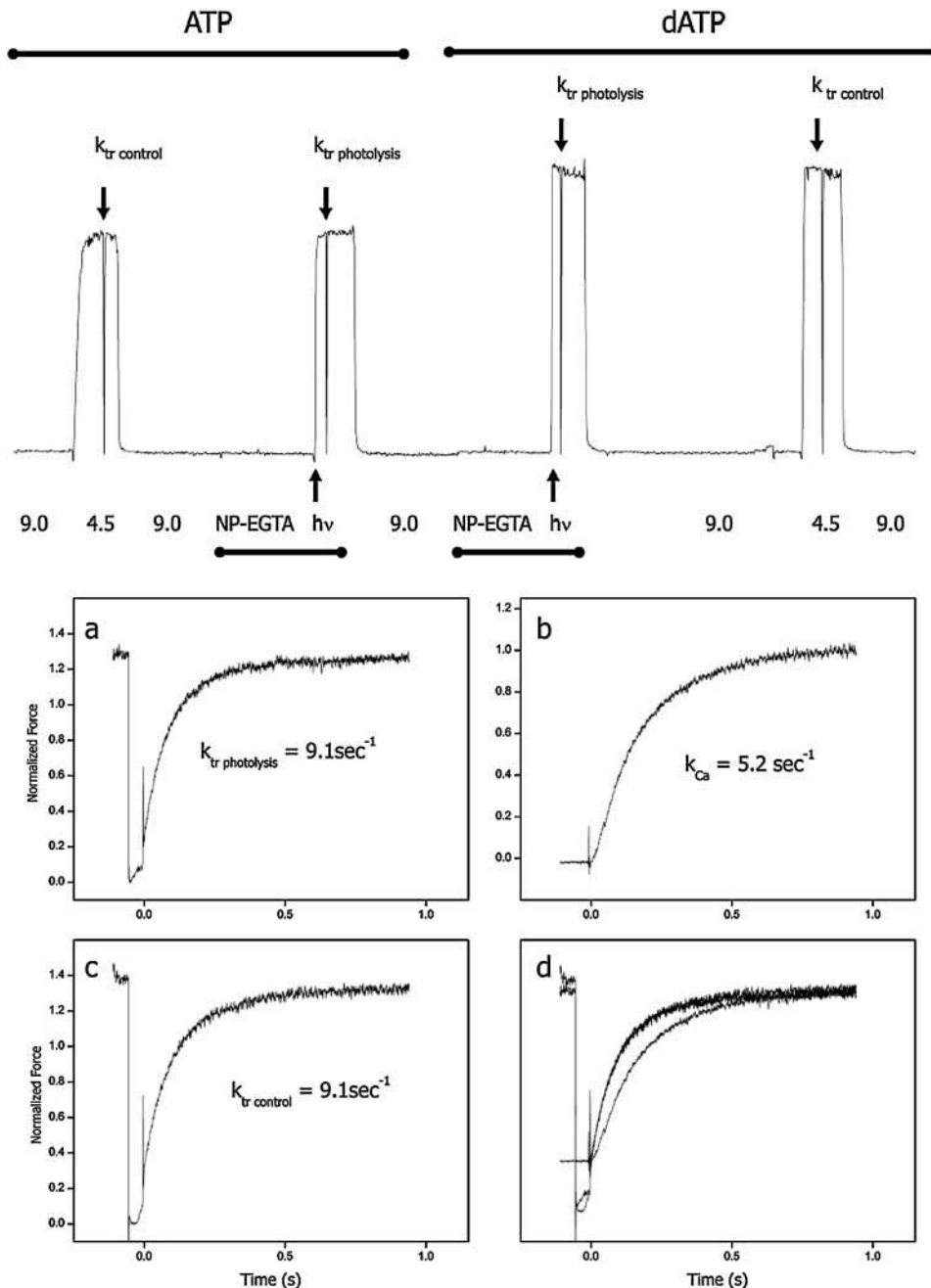


FIGURE 6 Procedure for sequential measurements of k_{Ca} and k_{tr} . (Upper panel) An example triton skinned rat trabeculae was transferred through a series of solutions containing 5 mM ATP (left) or dATP (right) with the indicated pCa (17°C). Where NP-EGTA is indicated the trabecula was transferred to a solution containing 2 mM NP-EGTA with 1.8 mM added Ca^{2+} , then activated by a pulse of laser light (arrow, hv). Measurements of k_{tr} were made at pCa 4.5 in control solution (k_{tr} control) and during maximal activation induced by photolysis of NP-EGTA (k_{tr} photolysis). k_{Ca} was determined from the time course of the transient after NP-EGTA photolysis. (Lower panel) Rapid time base recordings from upper panel. (a) k_{tr} photolysis for ATP determined immediately after activation by photolysis of NP-EGTA. (b) k_{Ca} induced by photolysis of NP-EGTA with ATP. (c) k_{tr} control for ATP. Rates are determined from the $t_{1/2}$ of the respective transients. (d) Force records from a and b superimposed to show relative rates.

solution (not shown) to confirm that photolysis produced maximal Ca^{2+} -activation conditions. The $k_{Ca} \rightarrow k_{tr}$ procedure was then repeated for activations with dATP as the contractile substrate (right side of Fig. 6, upper panel). The order of NTP testing was varied between preparations. Comparisons of k_{tr} and k_{Ca} were made only for maximal Ca^{2+} activation and then only if the photolysis of NP-EGTA produced Ca^{2+} activation within 5% of the force level in control activations. For this example trabecula it is obvious this is the case and that F_{max} was increased by dATP in both control and NP-EGTA activations.

Fig. 6, a–d, shows the k_{tr} and k_{Ca} measurements for ATP at greater resolution and demonstrates two consistent findings: 1), steady-state force and k_{tr} after activation by photolysis of NP-EGTA was similar to control activations; and 2), k_{Ca} was slower than k_{tr} . Fig. 7 shows higher time resolution examples of k_{Ca} and the subsequent k_{tr} force traces comparing activations with ATP versus dATP, demonstrating that both measurements are faster when dATP is the substrate for contraction. The data for all experiments is summarized in Table 2. The k_{tr} averaged 75% faster than k_{Ca} for ATP and 65% faster for dATP, suggesting that the relative difference

TABLE 2 Comparison of k_{Ca} and k_{tr} in the same maximal Ca^{2+} activation with ATP versus dATP as the contractile substrate

	ATP	dATP	dATP/ATP
k_{Ca}	4.3 ± 0.7 (8)	6.9 ± 0.4 (10)	1.61
k_{tr}	7.6 ± 0.6	11.4 ± 1.4	1.49

The number of trabeculae used for each NTP condition is given in parentheses. For most trabeculae, k_{Ca} and k_{tr} measurements were made for both ATP and dATP.

between the two measurements is independent of cross-bridge cycling rates. The data also show that dATP increased k_{tr} by 50% and k_{Ca} by 60% over measurements with ATP, suggesting faster cross-bridge cycling (dATP) enhances both rates by a similar mechanism.

DISCUSSION

There are three major findings from this study: 1), altering cross-bridge cycling by substitution of dATP for ATP as the contractile substrate increases the Ca^{2+} sensitivity of steady-state force (Fig. 1) and k_{tr} (Fig. 4) in rat cardiac muscle at 15°C, independent of myosin isoform; 2), both maximal Ca^{2+} -activated steady-state force and the rate of force development (k_{tr} , k_{Ca}) can be significantly increased by increased cross-bridge binding and/or cycling rate (Fig. 5); and 3), the maximal rate that force develops by a step increase in Ca^{2+} (k_{Ca}) is significantly slower than the maximal rate of force redevelopment (k_{tr}) during activations with saturating levels of Ca^{2+} . The maximal steady-state force and k_{tr} data agree with previous work in cardiac muscle where we demonstrated that dATP elevates maximal Ca^{2+} activated force, k_{tr} , unloaded shortening velocity and filament sliding velocity in vitro motility assays (Regnier et al., 2000b). These studies provide information about how altering cross-bridge cycling influences cross-bridge binding and thin filament activation at submaximal levels of Ca^{2+} in cardiac muscle. The results also allow us to compare results in cardiac muscle with previous work in fast and slow skeletal muscle, where we have extensively studied the effects of cross-bridge binding and cycling rates versus thin filament dynamics on the Ca^{2+} activation dependence of steady-state force and force development kinetics (Regnier et al., 1996, 1998, 1999, 2000a,b). Those studies provided strong evidence that thin filament dynamics are limiting to the level and rate of force development in skeletal muscle during submaximal Ca^{2+} activation.

The existing structural sequences and conservation information for myosin heavy chains offer no explanation about how dATP might increase F_{max} in cardiac muscle, but not in skeletal muscle (Regnier et al., 2000b). Indeed the increase in F_{max} with dATP may be peculiar to mammalian cardiac muscle in general, as we have also found similar results in chemically demembranated trabeculae from mouse and dog heart (unpublished observations). In previous studies we

suggested that dATP might increase the number of force-generating cross-bridges during submaximal Ca^{2+} activation in skeletal muscle (Regnier et al., 1998) and during maximal Ca^{2+} activation in cardiac muscle (Regnier et al., 2000b) by increasing one or more transition rates associated with cross-bridge attachment and force generation while increasing the rate of cross-bridge detachment to a lesser extent. In this article, we now propose this enhanced cross-bridge attachment leads to increased activation, which is apparent at submaximal Ca^{2+} in both muscle types and in maximal force in cardiac muscle.

Steady-state force

Proportional increases in steady-state force and stiffness were found at all levels of Ca^{2+} activation with dATP. This apparent increase of thin filament activation could result from recruitment of strongly bound force-generating cross-bridges, from an increase in force per cross-bridge, or both. Since dATP increases cross-bridge cycling rate, it suggests the cross-bridge detachment rate (g_{app}) is increased, which further implies the rate of strong cross-bridge attachment (f_{app}) must increase to a greater extent to provide a higher level of steady-state force and stiffness (Regnier et al., 2000b). This effect of dATP appears to be partially or completely independent of the overall cross-bridge cycling rate, as there were increases in both maximal force and Ca^{2+} sensitivity of force for trabeculae from untreated versus PTU-treated rats (Fig. 1; Table 1). This suggests the effects of dATP are largely independent of myosin kinetics (Figs. 3 and 4), implying that enhanced strong cross-bridge binding is the important contribution of dATP to increased activation. Whatever the detailed mechanism of force enhancement by dATP, the net result is an apparent elevation of thin filament activation at all levels of Ca^{2+} in cardiac muscle with either α - or β -MHC. Interestingly, dATP increased the Ca^{2+} sensitivity of force (pCa_{50}) for trabeculae with β -MHC twice as much as for trabeculae with α -MHC (0.26 vs. 0.13 pCa units, respectively; Fig. 1 and Table 1). This suggests enhanced cross-bridge binding and/or cycling rate may improve activation in cardiac muscle with slow myosin. Our steady-state force-pCa results (Fig. 1) contrast with those reported by Metzger et al. (1999) in their study of myocytes from untreated versus PTU-treated rats. Although we report no difference in the Ca^{2+} sensitivity of force (pCa_{50}) for trabeculae from untreated versus PTU-treated rats, they observed a decrease in pCa_{50} by 0.17 units with no change in the slope (Metzger et al., 1999). It is unclear why our results differ, but the focus and novel discovery of our study is that increased cross-bridge binding (with dATP) increases pCa_{50} of force for both untreated and PTU-treated trabeculae without changing the apparent cooperativity (n_H) of the force-pCa relationship (Fig. 1).

In cardiac muscle changes in cross-bridge binding have been shown to increase Ca^{2+} binding to troponin C (Hofmann and Fuchs, 1987; Wang and Fuchs, 1994; Martyn and

Gordon, 2001). Our experiments do not allow us to determine if enhanced Ca^{2+} sensitivity of force with dATP is correlated with increased Ca^{2+} binding to troponin C or via Ca^{2+} -independent increases in strong cross-bridge binding (or both). The increased force at saturating $[\text{Ca}^{2+}]$, however, is most likely explained by increased cross-bridge binding alone because of proportional increases in trabeculae stiffness. To determine if force enhancement by dATP directly results in a shifting of thin filament toward the “on” state will require a more direct measurement of the state of tropomyosin on thin filaments.

Rate of force development (k_{tr} , k_{Ca})

In this study the Ca^{2+} sensitivity of k_{tr} was increased with dATP (Fig. 4; Table 1), similar to steady-state force. When k_{tr} was plotted versus the steady-state force produced at varying $[\text{Ca}^{2+}]$, it was apparent that k_{tr} was similar at force and stiffness levels common to both substrates. However, with dATP both k_{tr} and force rose substantially above maximal levels for ATP for trabeculae from both untreated (Fig. 5 *a*) and PTU-treated (Fig. 5 *b*) rats, indicating the effect was independent of cardiac myosin isoforms (Regnier et al., 2000b). Changes in the force- k_{tr} relationship at high levels of Ca^{2+} with dATP could result from greater activation of thin filaments by cross-bridges, as suggested by the increased stiffness (Fig. 2). Evidence for elevation of the rate force develops during submaximal Ca^{2+} activation by enhanced cross-bridge binding was provided by Fitzsimons et al. (2001), who found that NEM S-1 binding to thin filaments at submaximal forces raised k_{tr} to levels measured during maximal Ca^{2+} activations. Since NEM S-1 cross-bridges are strong binding and slowly dissociating, they suggested that thin filaments with bound NEM S-1 were at a much higher activation level than in the absence of NEM S-1 at the same level of submaximal Ca^{2+} , thus allowing a faster rate of force development. An additional effect of dATP may be directly attributed to a faster rate of cross-bridge cycling with dATP since maximal k_{tr} is the sum of $f_{app} + g_{app}$, both of which could be increased by dATP (Regnier et al., 2000b). However, the similar effect of dATP on k_{tr} -force relations (Fig. 5; Table 1) for trabeculae from untreated (mostly α -MHC) and PTU-treated (β -MHC) rats indicates that cross-bridge cycling rate cannot be the only factor limiting the level and rate of force development in cardiac muscle. If k_{tr} at submaximal levels of activation is limited by the intrinsic acto-myosin NTPase rate of cross-bridges, we should have observed an elevation of k_{tr} at all levels of force. In contrast, Fig. 5 shows similar values of k_{tr} for ATP and dATP at the same level of force and stiffness, imply that a noncross-bridge cycling mechanism is limiting the rate that force can develop. An alternative interpretation of our results would be that the amount of force generated per cross-bridge is enhanced with dATP (compared with ATP). However, our previous observation that maximal Ca^{2+} -activated force is not elevated

by dATP in either fast or slow soleus skeletal muscle fibers argues against this. Slow skeletal fibers contain the same myosin isoform (β -MHC) present in trabeculae from PTU-treated rats, indicating dATP-induced elevation of force does not result from an increased level of force per cross-bridge. This suggests that the elevation of force and k_{tr} by dATP result from cross-bridge dependent enhancement of thin filament activation.

In cardiac muscle the relatively low Ca^{2+} -activation dependence of k_{tr} (a two- to fivefold change compared with a 10–15-fold change for skeletal muscle) could result from an intrinsically lower acto-myosin ATPase activity at maximal activation or a relative limitation of the level of thin filament activation, even at saturating $[\text{Ca}^{2+}]$. The last possibility is supported by the observations that 1), cardiac TnC (cTnC) has a single regulatory Ca^{2+} -binding site (Johnson et al., 1980) compared with the two regulatory Ca^{2+} -binding site for fast skeletal muscle; 2), cTnC undergoes less and slower conformational change upon Ca^{2+} -binding than skeletal TnC (Sia et al., 1997; Hazard et al., 1998); 3), the Ca^{2+} induced conformational change associated with TnI interaction is slow for cardiac TnC (perhaps as low as $4\text{--}6\text{ s}^{-1}$) (Dong et al., 1996, 1997); and 4), when cardiac TnC is fully substituted for endogenous TnC in skeletal fibers maximum force is significantly lower (Chase et al., 1994; Morris et al., 2001). An additional consideration is that the signal transmission of Ca^{2+} binding to TnC to tropomyosin could be slow relative to cross-bridge cycling rates, especially at low Ca^{2+} concentrations. If the level or rate of thin filament activation is limited in cardiac muscle, interventions that elevate thin filament activation might be expected to enhance both maximum force and k_{tr} , as we observe with dATP. We have previously demonstrated that thin filament activation dynamics are rate limiting to force development at low levels of Ca^{2+} in skeletal fibers (Chase et al., 1994; Regnier et al., 1998). We now suggest this mechanism may also apply to cardiac muscle.

This hypothesis is further supported by comparisons of k_{tr} and k_{Ca} during the same activation of trabeculae at pCa 4.5. For both ATP and dATP k_{Ca} was consistently only about half as fast as k_{tr} (Table 2, Figs. 6 and 7), indicating the dynamics of thin filament activation by Ca^{2+} limit the rate of force development. However, because force develops from different levels during k_{tr} and k_{Ca} measurements, differences in time courses of force rise could be influenced by the distribution of compliance between cross-bridges and noncross-bridge components (Huxley et al., 1994; Luo et al., 1994; Higuchi et al., 1995; Martyn et al., 2002). Comparison of the force traces in Fig. 7 indicates that the k_{tr} force redevelops from a nonzero value, whereas it rises from relaxed levels after the Ca^{2+} transient. As a result, in k_{tr} measurements the compliance of noncross-bridge structures would be high relative to cross-bridge compliance at the beginning of force development, resulting in an apparent slowing of force kinetics (Luo et al., 1994; Martyn et al., 2002) and thus would

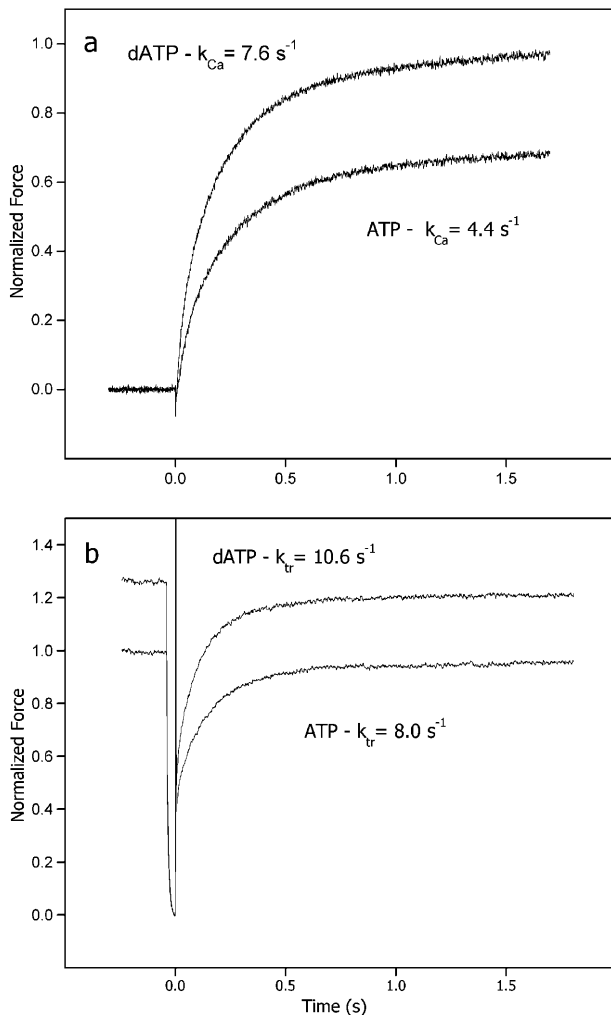


FIGURE 7 Comparison of k_{Ca} and k_{tr} photolysis for ATP versus dATP from an untreated rat trabecula. The nucleotide and rate determined from $t_{1/2}$ are shown next to each trace. (a) k_{Ca} . (b) k_{tr} photolysis.

underestimate the difference between k_{tr} and k_{Ca} . In comparison, during k_{Ca} measurements noncross-bridge compliance would be lowest at the beginning of force rise, thus the initial rate of force development would be faster, slowing as cross-bridge compliance decreases with increasing force. However, there was no indication of an initial rapid rise of force that approached k_{tr} during k_{Ca} measurements. Thus differences in distribution of compliance between cross-bridge and noncross-bridge components do not explain the different time course of k_{tr} and k_{Ca} .

If cross-bridge cycling kinetics were limiting the rate of force development, we would expect k_{Ca} and k_{tr} to be similar. The thin filament activation state should be in near equilibrium during tension redevelopment with the k_{tr} protocol. Changes in myoplasmic $[Ca^{2+}]$ during tension transients (Vandenboom et al., 1998; Wang and Kerrick, 2002) or Ca^{2+} binding when force is inhibited in cardiac muscle (Wang and Fuchs, 1994; Martyn and Gordon, 2001)

indicate that some Ca^{2+} dissociates from thin filaments during the release-retch transients of k_{tr} measurements. However, this amount is likely to be a small fraction of Ca^{2+} bound to troponin C at pCa 4.5 (Martyn and Gordon, 2001). Furthermore, any thin filament deactivation during the release phase of the release-retch transient would lead to a slower value of maximal k_{tr} , and thus underestimate differences between k_{tr} and k_{Ca} . In contrast, force development during the k_{Ca} measurement begins with near zero levels of Ca^{2+} bound to thin filaments. Thus our observation that k_{Ca} is always slower than k_{tr} supports a hypothesis that thin filament activation by Ca^{2+} is rate limiting for force development even at saturating $[Ca^{2+}]$. Previously Palmer and Kentish (1998) reported no difference between k_{act} (reported here as k_{Ca}) and k_{tr} in rat and guinea pig trabeculae at 22°C. However these measurements were not obtained during the same activation and may have been from separate trabeculae. Additionally, their k_{act} values in rat trabeculae varied almost fourfold with some activations producing more force than the control activations, and maximum k_{tr} varied by more than twofold. This suggests some of their measurements may have been made in submaximally activating conditions. The argument that thin filament regulatory protein dynamics limit the rate that force develops in cardiac muscle is further supported by the observation that both rates were elevated ~60% by dATP, whereas k_{Ca} remained slower than k_{tr} (Table 1). Thus, even though cross-bridge cycling kinetics can be increased by dATP, the kinetics of thin filament activation by Ca^{2+} may limit the rate force develops in rat cardiac muscle. If so, mechanisms that enhance cross-bridge binding at a given submaximal level of intracellular Ca^{2+} , such as phosphorylation of myosin-binding protein C could be important in increasing the power output of the heart during sympathetic stimulation. This mechanism would not be available to skeletal muscle, where Ca^{2+} binding to TnC appears to be much more successful in fully activating thin filaments and where contraction usually results from action potential trains that allow muscles to contract at the upper end of the force-pCa relationship.

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