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Effect of Ca^{2+} on weak cross-bridge interaction with actin in the presence of adenosine 5′-[γ-thio]triphosphate

(weak-binding cross-bridge states/strong-binding cross-bridge states/actin binding kinetics/nucleotide saturation/nucleotide affinity)

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ABSTRACT **In the presence of the nucleotide analog adenosine 5′-[γ-thio]triphosphate (ATP[γS]), effects of Ca^{2+} on stiffness and equatorial x-ray diffraction patterns of single skinned fibers of the rabbit psoas muscle were studied. It is shown that cross-bridges in the presence of ATP[γS] have properties of the weak-binding states of the ATP hydrolysis cycle. Raising the Ca^{2+} concentration up to pCa 4.5 has little effect on actin affinity of cross-bridges in the presence of ATP[γS]. However, the rate constants for cross-bridge dissociation and reassociation from and to actin are reduced by about 2 orders of magnitude. In addition, nucleotide affinity of the cross-bridge is much smaller at high Ca^{2+} concentrations. Implications for interpretation of fiber stiffness recorded during isotonic shortening and the rising phase of a tetanus are discussed.**

It is generally accepted that muscle contraction is driven by cyclic interactions between the heads of the myosin molecules (cross-bridges) and actin while ATP is hydrolyzed. In muscle fibers, as in solution (1–3), the biochemical states of the cross-bridge cycle can be broadly divided into two groups: the weak-binding states and the strong-binding states (4–7). In the weak-binding states, cross-bridges are characterized by their low and ionic strength-dependent affinity for actin and, most specifically, by their inability to activate the contractile system. However, cross-bridges that are weakly attached to actin are found to be necessary precursors to force generation (8, 9). The strong-binding states are characterized by much higher actin affinity under comparable conditions and have been proposed to be the main force-generating states (10). Finally, in both the weak- and the strong-binding states, cross-bridges can attach and detach to and from actin rapidly regardless of the actin affinity, although the rate constants for dissociation and reassociation differ, depending on the cross-bridge states (4–7, 11).

It is well known that muscle contraction is regulated by Ca^{2+}. We have previously shown that Ca^{2+} controls the rate constant of force redevelopment, which is thought to be associated with the transition of cross-bridges from the weak- to the strong-binding states (12). The mechanism of this Ca^{2+} effect on cross-bridge turnover kinetics is not yet clear. We proposed that Ca^{2+} regulation of force redevelopment may be mediated by a Ca^{2+}-dependent equilibrium between a "turned on" and "turned off" form of the actin filament, with phosphate-release reactions only occurring when the actin filament is in the turned on form (12). Alternatively, Ca^{2+} effects on actin affinity of the weak-binding (preforce-generating) states may cause Ca^{2+} regulation of force redevelopment. To test this latter possibility, we studied the effects of Ca^{2+} on the weak cross-bridge binding to actin, particularly effects on actin affinity of cross-bridges and on their kinetics for association and dissociation to and from actin.

In muscle fibers, Ca^{2+} effects on the weak cross-bridge binding to actin cannot be investigated in the presence of the physiological nucleotide MgATP, since active cross-bridge turnover would occur. In that case, cross-bridges are distributed in both weak- and strong-binding states such that the properties of weak-binding states cannot be evaluated unambiguously. To bypass this problem, we used the slowly hydrolyzable nucleotide analogue adenosine 5′-[γ-thio]-triphosphate (ATP[γS]) in place of MgATP.

In this report, we first ensure that MgATP[γS]·cross-bridges exhibit properties characteristic of the weak-binding states. Secondly, we show that Ca^{2+} has little effect on the actin affinity of these ATP[γS]·cross-bridges. However, the rate constants for association and dissociation to and from actin are strongly Ca^{2+} dependent as is the affinity of the cross-bridge for nucleotide. A preliminary account of this work has been presented (13).

MATERIALS AND METHODS

Fiber Preparation. Rabbit psoas muscle fibers were chemically skinned with Triton X-100 according to the method described earlier (14–16). Single fibers were prepared and mounted as described (14, 17).

Solutions. The standard experimental solutions at low Ca^{2+} concentration (pCa ≈ 8) contained 10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 1 mM dithiothreitol, and 0.2 Ap_5A. MgATP[γS], hexokinase, and glucose were added to this solution as required. Hexokinase and glucose were added to reduce even traces of contaminant ATP still present after purification (see below). ATP[γS], hexokinase, and glucose were added in fixed proportions, reaching 0.5 unit of hexokinase per ml and 200 mM glucose for 10 mM MgATP[γS]. For high Ca^{2+} concentrations, EGTA was replaced by CaEGTA. Ionic strength (μ) was adjusted by adding potassium propionate. For experiments in the presence of MgATP, neither hexokinase nor Ap_5A was added to the solutions.

Purification of ATP[γS]. ATP[γS] was purified by ion-exchange chromatography to remove AMP, ADP, and ATP. ATP[γS] (Boehringer Mannheim) was eluted from a DEAE-Sephadex column (A 25; Pharmacia) with a 150–500 mM gradient of LiCl (Merck) at 4°C. The ATP[γS] fraction was dissolved in 10 vol of a 2:1 (vol/vol) mixture of acetone and ethanol. The precipitated ATP[γS] was washed with ethanol, dried, and stored at −20°C. Concentration and purity of the ATP[γS] were checked by HPLC analysis. ADP could be reduced to 2–3%; other nucleotides were not detectable.

Mechanical Apparatus and X-Ray Diffraction. Equipment for recording of fiber stiffness and equatorial x-ray diffraction patterns has been described (15, 17, 18).

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Abbreviation: ATP[γS], adenosine 5′-[γ-thio]triphosphate.

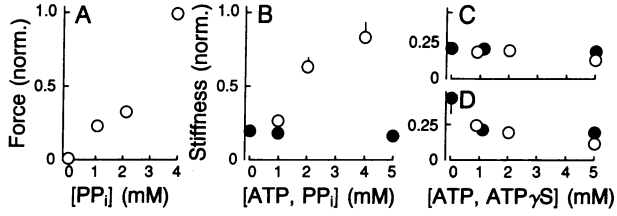

FIG. 1. Effect of adding increasing amounts of nucleotide in the presence of a low concentration of MgATP. (*A*) Active force generation after adding $MgPP_i$ to a solution containing 150 μM MgATP, which (without an ATP backup system) is just sufficient to keep the fiber fully relaxed under the given temperature and ionic strength conditions. The abscissa indicates the amount of $MgPP_i$ added. The temperature was 5°C; the ionic strength was 56 mM. At 4 mM $MgPP_i$, the active force reaches about 10% of the maximum isometric force (data from one fiber). norm., normalized. (*B*) Fiber stiffness when adding MgATP (●) or $MgPP_i$ (○) to solution with 150 μM MgATP. The conditions were the same as those in *A*. (*C*) Fiber stiffness when adding MgATP[γS] (○) or MgATP (●) to a solution containing 150 μM MgATP. (*D*) Same as *C*, but the experimental temperature was raised to 10°C at which temperature 150 μM MgATP was no longer sufficient to keep the fiber fully relaxed, as seen by elevated fiber stiffness (at 0 mM additional nucleotide), which decreases to the fully relaxed value upon addition of either MgATP (●) or MgATP[γS] (○). Fiber stiffness was measured by ramp-shaped stretches of 5×10^3 nm per half-sarcomere per s and normalized to the stiffness observed in the presence of 150 μM ATP at 5°C. (*B–D*) Values are the means ± SEM (n = 4).

Fiber stiffness was measured by applying ramp-shaped stretches at velocities of 10^{-1} to 5×10^5 nm per half-sarcomere per s. Fiber stiffness was defined as the ratio of force increment over filament sliding when filament sliding had reached 2 nm per half-sarcomere (chord stiffness).

The ratio of the intensities of the two innermost equatorial reflections [1, 0] and [1, 1] was calculated from the integrated intensities of these two reflections. An increase in this ratio is expected if a transfer of mass occurs from the thick filament region to the thin filament region—for example, when cross-bridges attach to actin (19, 20).

Before measuring stiffness and x-ray diffraction patterns in the presence of MgATP[γS], fibers were incubated in rigor solution, which was renewed two times to remove contaminating MgATP.

RESULTS

Characterization of Cross-Bridges in the Presence of ATP[γS]. For studying the effects of Ca^{2+} on weak cross-bridge interactions with actin, we first demonstrated that cross-bridges in the presence of ATP[γS] represent weak-binding cross-bridges.

Weak-binding cross-bridges are most clearly distinguished from strong-binding cross-bridges by their inability to activate the contractile system (in the absence of Ca^{2+}), whereas strong-binding cross-bridges can do so (e.g., refs. 21 and 22). This difference remains even when actin affinities are matched by proper adjustment of ionic strength. We therefore tested whether cross-bridges in the presence of ATP[γS] can activate the contractile system, similar to cross-bridges in the presence of pyrophosphate (PP_i), which are known from biochemistry to represent strong-binding cross-bridge states (22, 23).

In such a test, it has to be ensured that nucleotide saturation does not change. Otherwise, activation may simply result from an increasing fraction of nucleotide-free cross-bridges that are known to activate the contractile system (21, 22).

The general approach for such testing is shown in Fig. 1. In the absence of $MgPP_i$ or MgATP[γS], the MgATP concentration (0.15 mM, no ATP backup) was adjusted to the lowest possible value necessary to keep the contractile system fully relaxed (low fiber stiffness, no active force). By adding increasing amounts of $MgPP_i$, the fractions of nucleotide-free cross-bridges and MgATP·cross-bridges decrease while the fraction of $MgPP_i$·cross-bridges increases. Addition of increasing amounts of $MgPP_i$ results in (*i*) development of an increasing amount of active force (Fig. 1*A*) and (*ii*) in an increase in fiber stiffness up to the values seen with $MgPP_i$ in the presence of Ca^{2+} (Fig. 1*B*). Both effects indicate activation of the contractile system, confirming that $MgPP_i$·cross-bridges represent strong-binding states.

Fig. 1*C* shows the same experiment but with MgATP[γS] instead of $MgPP_i$. Upon addition of ATP[γS], neither an increase in fiber stiffness nor active force is detectable. To rule out that this is due to lack of binding of ATP[γS] to the cross-bridges, the contractile system was first partially activated either by a further reduction in the MgATP concentration (data not shown) or by an increase in temperature (Fig. 1*D*), which resulted in increased fiber stiffness and steady active force. Subsequent addition of MgATP[γS] resulted in full relaxation (decrease in fiber stiffness, no more active force). Thus, MgATP[γS] binds to the cross-bridge under these conditions, but the increase in the number of ATP[γS]·cross-bridges does not activate but rather relaxes the contractile system, similar to addition of MgATP.

The results of Fig. 1 suggest that, in terms of activation level of the contractile system, MgATP[γS]·cross-bridges have properties similar to MgATP·cross-bridges (weak-binding cross-bridges) but are different from $MgPP_i$·cross-bridges (strong-binding cross-bridges).

Comparison of the stiffness–speed relation at low Ca^{2+} concentration (pCa 8) in the presence of MgATP[γS] with that in the presence of MgATP (Fig. 2*A*) and comparison of equatorial diffraction patterns under the same conditions (Fig. 2*B*) show that at pCa 8 cross-bridges show almost identical properties for the two nucleotides. Thus, cross-bridges in the presence of MgATP[γS] show all the characteristic properties of weak-binding states.

Saturation with ATP[γS] at High and Low Ca^{2+} Concentrations. Solution studies showed that binding of Ca^{2+} to regulated actin increases the rate of dissociation of MgATP from myosin by 10- to 20-fold (24). This suggests that nucleotide saturation of cross-bridges is Ca^{2+} dependent. Therefore, we studied what concentrations of MgATP[γS] are required in both the absence and presence of Ca^{2+} for full

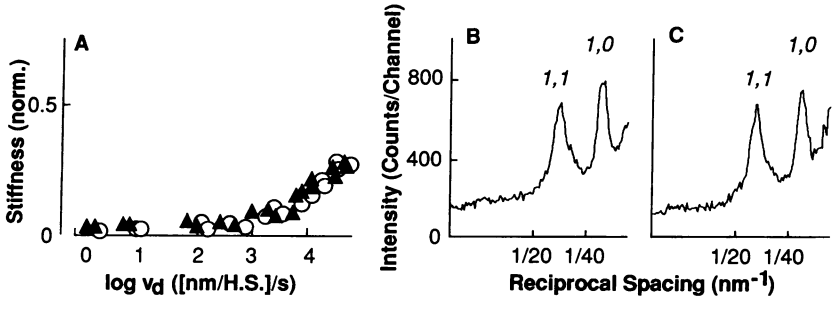

FIG. 2. Stiffness–speed relation and equatorial diffraction pattern in the presence of MgATP vs. MgATP[γS] at low Ca^{2+} (pCa 8). (*A*) Stiffness–speed relation in the presence of 1 mM MgATP (▲) or 1 mM MgATP[γS] (○) at μ = 30 mM and t = 1°C. Equatorial diffraction patterns in the presence of 10 mM MgATP (*B*) or 10 mM MgATP[γS] (*C*) at μ = 75 mM and t = 1°C are shown. norm., normalized.

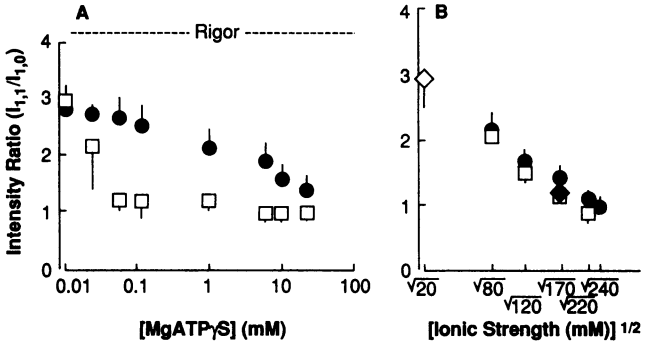

FIG. 3. (A) Nucleotide saturation at high and low Ca^{2+} concentrations. Nucleotide binding is characterized by the decrease in the I_{11}/I_{10} ratio of equatorial x-ray diffraction patterns. □, pCa 8; ●, pCa 4.5; μ = 170 mM, t = 1°C. Data are the means ± SEM of four fibers. (B) Effect of ionic strength on the I_{11}/I_{10} ratio of fibers in the presence of 10 mM MgATP[γS]. □, pCa about 8; ●, pCa 4.5. For comparison, the I_{11}/I_{10} ratio in the presence of 10 mM MgATP at pCa 8 and μ = 170 mM (◆) and in the presence of 1 mM MgATP at pCa 8 and μ = 20 mM (◇; cf. refs. 15 and 17) are given. Data are means ± SEM of eight fibers. Differences between high and low Ca^{2+} are statistically significant for ionic strengths of 120, 170, and 220 mM ($P < 0.01$).

nucleotide saturation of cross-bridges in the skinned fiber system. Equatorial x-ray diffraction patterns were recorded at various nucleotide concentrations. At μ = 170 mM, the intensities of the two innermost equatorial reflections I_{10} and I_{11} depend on the distribution of cross-bridges between the mostly detached weak-binding ATP[γS] state and the actin-attached nucleotide-free state (15, 17). In Fig. 3A, the equatorial intensity ratio I_{11}/I_{10} of the equatorial diffraction patterns is plotted vs. MgATP[γS] concentration at low (pCa 8) and high Ca^{2+} (pCa 4.5) concentrations. With increasing MgATP[γS] concentrations, the I_{11}/I_{10} ratio decreases. However, there is a large difference in sensitivity to ATP[γS] at high and low Ca^{2+}. At low Ca^{2+} (pCa 8), no further change in the I_{11}/I_{10} ratio is observed above 0.05 mM MgATP[γS]. At high Ca^{2+} and at 10–20 mM MgATP[γS], the I_{11}/I_{10} ratio has not reached a completely nucleotide-independent value.‡ With increasing temperature, the saturation level becomes even lower (data not shown).

Effect of Activation on Actin Affinity of Cross-Bridges in the Presence of MgATP[γS]. Ca^{2+} effects on the fraction of cross-bridges attached to actin were investigated at 10 mM MgATP[γS] and low temperature to maximize nucleotide saturation. The fraction of cross-bridges bound to actin was characterized by recording the equatorial intensity ratio I_{11}/I_{10}. An increase in the fraction of cross-bridges attached to actin is expected to increase the I_{11}/I_{10} ratio. Fig. 3B shows the I_{11}/I_{10} ratio both at high and low Ca^{2+} concentrations recorded between μ = 80 and 240 mM. It has been estimated that over this range the fraction of attached weak-binding cross-bridges decreases from near 100% to as low as some 5% (6). Ca^{2+}, however, causes only a small increase in I_{11}/I_{10} throughout the entire range of ionic strengths used. This suggests that even when the fraction of cross-bridges attached to actin is significantly below 100%, Ca^{2+} causes only a small increase in this fraction in the presence of saturating concentrations of MgATP[γS].

Assuming full nucleotide saturation at both high and low Ca^{2+}, the maximum change in actin affinity of weak-binding cross-bridges with Ca^{2+} can be estimated if we assume no significant change in the overall configuration of weakly attached cross-bridges with ionic strength or Ca^{2+}. The change in I_{11}/I_{10} with Ca^{2+} can then be compared with the changes brought about by ionic strength (Fig. 3B). Raising Ca^{2+} at an ionic strength of 170 mM, an increase in I_{11}/I_{10} is observed that is comparable to that resulting from reduction of ionic strength (at pCa 8) from 170 mM to 120 mM. This change in ionic strength, however, is known to increase actin affinity of weak-binding cross-bridges 2- to 5-fold (25). Thus, the upper limit for an increase in actin affinity of ATP[γS]-cross-bridges with Ca^{2+} is 2- to 5-fold, which is in agreement with biochemical data on isolated proteins in solution (refs. 26–28; A. Resetar and J. M. Chalovich, personal communication). Any nucleotide-free cross-bridges at high Ca^{2+} will cause a higher I_{11}/I_{10} ratio. Thus, the above estimated increase in actin affinity is likely an overestimate.

Effect of Activation on Actin Binding Kinetics of Cross-Bridges in the Presence of MgATP[γS]. We previously demonstrated that plots of fiber stiffness vs. speed of imposed length changes (stiffness–speed relations) may be used to estimate kinetics of the dynamic actin interaction of cross-bridges (29, 30). Fig. 4A shows stiffness–speed relations recorded in the presence of 10 mM MgATP[γS] both at low and high Ca^{2+}. At low Ca^{2+}, the apparent fiber stiffness is very low over a wide range of speeds and increases significantly only for stretches faster than 10^3 nm per half-sarcomere per s. This stiffness–speed relation is indistinguishable from that recorded in the presence of MgATP under the same experimental conditions (Fig. 2A). At high Ca^{2+}, the stiffness–speed relation is shifted to the left (i.e., to slower stretch velocities) by more than 2 orders of magnitude.

To rule out that the difference in the stiffness–speed relation is due to accumulation of strong-binding states by active turnover at high Ca^{2+} concentrations, we have compared the steady-state rate of ATP[γS] hydrolysis in the presence of Ca^{2+} with that in the absence of Ca^{2+}. There is no detectable difference even after several hours of incubation (unpublished results), suggesting no detectable change in cross-bridge turnover in the presence of ATP[γS] with Ca^{2+}.

The shift of the stiffness–speed relation to slower stretch velocities by about 2 orders of magnitude suggests that kinetics of dynamic actin interaction of cross-bridges in the presence of ATP[γS] are Ca^{2+} dependent; specifically, the rate constant for cross-bridge detachment, k^-, may be reduced (29, 30). This possibility, however, has to be distinguished from effects on stiffness–speed relations arising from incomplete nucleotide saturation in the presence of Ca^{2+}. To do so, we performed quantitative simulations of stiffness–speed relations recorded under different conditions (e.g., data of Fig. 4). Data obtained at low Ca^{2+} and full nucleotide saturation (10 mM ATP[γS]; □ in Fig. 4A) can quantitatively be accounted for with a detachment rate constant of 4×10^4 s^{-1} and an equilibrium constant for binding to actin of 1. The dashed line in Fig. 4A represents the contribution of a 13–15% population of nucleotide-free cross-bridges at high Ca^{2+} (average increase in I_{11}/I_{10} with Ca^{2+}; compare with Fig. 3). The dotted line is the stiffness–speed relation expected for a population of 13–15% nucleotide-free cross-bridges and 85–87% ATP[γS]-cross-bridges with the same binding kinetics as in the absence of Ca^{2+}. To satisfactorily fit the experimental

‡It may be argued that at high concentrations of ATP[γS] our observations may be affected by traces of other nucleotides (e.g., ATP and ADP) that may still be present even after purification. However, the presence of even traces of ATP is detectable by signs of activation in the presence of Ca^{2+} (e.g., sarcomere movement and active force). In the presence of an adequate hexokinase/glucose system, activation of the contractile system was never detected in the presence of ATP[γS]. Hexokinase at 0.5 unit/ml was found to be low enough not to cause significant breakdown of ATP[γS] during the experiment (monitored by HPLC). To ensure sufficient reduction of contaminant ATP by the hexokinase/glucose system, the necessary glucose/ATP[γS] ratio was found to be ≥10:1. Significant effects of ADP on our measurements were ruled out both by lowering the ADP contamination by adding apyrase (Sigma; grade VIII, up to 50 units/ml) and by adding ADP to the ATP[γS]-containing solutions (up to an ADP/ATP[γS] ratio of at least 1:2). Both interventions had no effect on our observations.

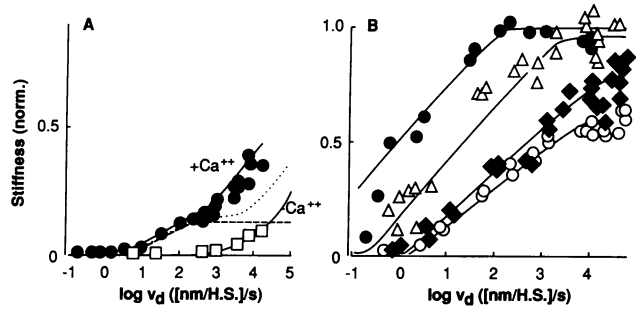

FIG. 4. (*A*) Stiffness–speed relations for 10 mM MgATP[γS] at pCa 4.5 (●) or pCa 8 (□). Ionic strength was 75 mM. Temperature was −3°C, to minimize effects from incomplete nucleotide saturation at high Ca^{2+}; x-ray diffraction data like those in Fig. 3*A* showed that lowering the temperature increases nucleotide saturation (unpublished results). Symbols represent average values from four to seven fibers. Data are normalized (norm.) to rigor stiffness. Solid, dashed, and dotted lines are stiffness–speed relations expected from quantitative modeling. For this modeling, the free energy functions AM and MT/AMT of Eisenberg and Greene (ref. 31; modified according to ref. 30) were used for the nucleotide-free (AM) and ATP[γS]·cross-bridges, respectively. Calculation of the stiffness–speed relations followed the procedure of Schoenberg *et al.* (32). The difference between I_{11}/I_{10} in the presence of 10 mM ATP[γS] at high and low Ca^{2+} (Fig. 3*A*) was used as a measure for the fraction of cross-bridges in the nucleotide-free AM state. The solid line ($-Ca^{++}$) is the stiffness–speed relation expected for low Ca^{2+} and full nucleotide saturation (10 mM ATP[γS]), with detachment a rate constant of $4 \times 10^4\ s^{-1}$ and an equilibrium constant for binding to actin of 1. The dashed line is the maximum possible contribution from incomplete saturation in the presence of Ca^{2+} (13–15% nucleotide-free cross-bridges). The dotted line is the expected stiffness–speed relation for a population of 13–15% nucleotide-free cross-bridges plus 85–87% ATP[γS]·cross-bridges with the same actin binding kinetics as in the absence of Ca^{2+}. The solid line ($+Ca^{++}$) is the stiffness–speed relation when assuming 13–15% nucleotide-free cross-bridges and the remaining 85–87% ATP[γS]·cross-bridges with rate constants of detachment and reattachment from and to actin 20-fold slower than assumed for low Ca^{2+}. (*B*) Stiffness–speed relations recorded in the presence of different concentrations of ATP[γS] at 30°C where nucleotide saturation is less than that at low temperature. The ATP[γS] concentration was 0.1 mM (●), 1 mM (△), 5 mM (◆), or 10 mM (○). The conditions were pCa = 4.5 and μ = 75 mM. Stiffness was normalized to rigor stiffness. Note that the ATP[γS] concentration affects the position of the curves along the abscissa, which was shown to be a measure of the rate constant of cross-bridge dissociation from actin (29, 30). Nucleotide dependence of the apparent rate constant for cross-bridge dissociation from actin indicates that, at least up to 5 mM ATP[γS], for some cross-bridges dissociation from actin involves nucleotide-binding reaction; i.e., some myosin heads are free of nucleotide. v_d, velocity of stretch; H.S., half-sarcomere.

data in the presence of Ca^{2+}, with 13–15% of nucleotide-free cross-bridges in the population, the rate constant for dissociation of the ATP[γS]·cross-bridges from actin has to be reduced about 20-fold; if the fraction of nucleotide-free cross-bridges is insignificant, it has to be reduced about 100-fold.

DISCUSSION

The main findings of this paper are as follows.

(*i*) ATP[γS]·cross-bridges, as judged from their inability to activate the contractile system, represent analogues of the weak-binding states of the cross-bridge cycle. The previously reported activation of the contractile system in the presence of ATP[γS] (33) was due to incomplete saturation of cross-bridges with ATP[γS] under conditions such as high temperature and high Ca^{2+}.

(*ii*) Activation has a large effect on ATP[γS] saturation. In the absence of Ca^{2+}, saturation is reached at 0.05–0.1 mM ATP[γS]. The steep increase of I_{11}/I_{10} at lower concentrations, up to the level observed in the presence of Ca^{2+}, suggests that nucleotide-free cross-bridges activate the contractile system, resulting in a similar affinity of cross-bridges for nucleotide as at high Ca^{2+}. In the presence of Ca^{2+}, the saturation level is titrated over a concentration range of more than 3 orders of magnitude (Fig. 3*A*). This broad titration can be accounted for by strain-dependent nucleotide binding (34) and a wide range of strain experienced by attached cross-bridges in muscle due to mismatch between actin and myosin periodicities (H.J.K., unpublished results). The effect of Ca^{2+} on affinity of cross-bridges for nucleotide seems to be a general feature. For example, our preliminary results suggest that binding of GTP and 2-[(4-azido-2-nitrophenyl) amino]propyl triphosphate (kindly provided by R. Yount Washington State University) to cross-bridges in skinned fibers is similarly affected by Ca^{2+}.

(*iii*) At high nucleotide saturation (about 100% at low Ca^{2+}, at least 85% at high Ca^{2+}), Ca^{2+} has only little effect on actin affinity of cross-bridges. The estimated 2- to 5-fold increase (Fig. 4*B*) is an upper limit since part of the increase in the I_{11}/I_{10} ratio with Ca^{2+} is due to incomplete saturation even for 10 mM ATP[γS] at 1°C (Fig. 3*A*; note the decrease in I_{11}/I_{10} between 10 and 20 mM ATP[γS]).

(*iv*) Ca^{2+} reduces actin binding kinetics of MgATP[γS]·cross-bridges, specifically the rate constant for cross-bridge dissociation. The observed shift in the stiffness–speed relation with Ca^{2+} to slower stretch velocities by about 2 orders of magnitude (Fig. 4*A*) cannot be accounted for by reduced nucleotide saturation at the high Ca^{2+} alone (Fig. 4*A*, dotted line). Instead, the shift of the stiffness–speed relation suggests a 20- to 100-fold reduction in the rate constant for dissociation of the ATP[γS]·cross-bridge from actin when the Ca^{2+} concentration is raised.

Since actin affinity of the nucleotide cross-bridge (k^+/k^-) is little affected by Ca^{2+}, the rate constant for reassociation of the ATP[γS]·cross-bridge apparently is also reduced (between 20- and 100-fold) when Ca^{2+} is raised.

Implications for Regulation of Muscle Contraction. It was demonstrated previously that the predominant regulatory effect of Ca^{2+} is through modulation of turnover kinetics of cross-bridges (12). In particular, it was shown that Ca^{2+} has a marked effect on the rate constant, f_{app}, for the transition from weak- to strong-binding states (12). It is demonstrated in this study that there is at most a 2- to 5-fold increase in actin affinity of weak-binding cross-bridges with Ca^{2+}. This small effect cannot account for the more than 10- to 20-fold increase in f_{app} upon full activation by Ca^{2+}. Similarly, the slowing of the association and dissociation kinetics of weak-binding cross-bridges from and to actin with Ca^{2+} does not provide a straightforward account for the increase in f_{app}. Instead, it appears more likely that Ca^{2+} affects force redevelopment by the mechanism that we previously proposed—i.e., by Ca^{2+} affecting the equilibrium between the turned on and turned off form of the actin filament, with P_i release reactions only occurring when cross-bridges are attached to the turned-on form of actin (12).

Relationship to Previous Studies. Dantzig *et al.* (35) reported a large Ca^{2+} effect on actin affinity of cross-bridges in the presence of MgATP[γS]. The experiments of Dantzig *et al.* (35) were done in the presence of 1 mM MgATP[γS] at 20°C with an ionic strength of 200 mM. In agreement with Dantzig *et al.* (35), we also find a large increase in fiber stiffness with Ca^{2+} under these conditions. However, our experiments show that at high temperature in the presence of 1 mM MgATP[γS] Ca^{2+} has a large effect on nucleotide saturation level. Our control experiments (Fig. 4*B*) indicate that the observed increase in stiffness with Ca^{2+} under these conditions results from the leftward shift of the stiffness–speed relation due to an increasing fraction of nucleotide-free cross-bridges (Fig. 4*B*), in addition to the shift due to the

reduction of the rate constant for dissociation of the ATP[γS]·cross-bridge with Ca^{2+} (Fig. 4*A*).

The Ca^{2+} effects of Baker and Cooke (36) on fiber stiffness and EPR spectra were recorded in the presence of 1 mM ATP[γS] at 5°C. Again, this concentration is insufficient for full nucleotide saturation in the presence of Ca^{2+}. The reported stiffness data are consistent with our data (Fig. 4*B*; note that for ATP[γS] concentrations that result in incomplete saturation, fiber stiffness is not expected to be speed independent as in rigor). The EPR spectra of Baker and Cooke (36) reveal the decrease in nucleotide saturation with Ca^{2+} (15% ordered, rigor-type probes). The difference in the percentage of ordered probes (15%) vs. magnitude of stiffness (50% rigor stiffness; ref. 36) can be accounted for by (*i*) contribution to stiffness by ATP[γS]·cross-bridges and (*ii*) the observation that EPR spectra change when one head of a myosin molecule binds nucleotide, whereas fiber stiffness only changes when both heads bind the nucleotide (37, 38).

Implications for Cross-Bridge Action in Muscle. By assuming that the effects of Ca^{2+} on actin binding kinetics and nucleotide saturation seen in the presence of ATP[γS] also apply, at least qualitatively, for weak-binding cross-bridge states in the presence of MgATP,[§] these observations have several implications for our understanding of the processes during isotonic shortening and the rising phase of a tetanus.

For isotonic steady-state shortening, the previously reported decrease in apparent fiber stiffness to 40% (frog fibers; refs. 39 and 40) or 15% (rabbit fibers; ref. 41) of isometric fiber stiffness may require reinterpretation. A leftward shift of the stiffness–speed relation for the weak-binding cross-bridge states at high Ca^{2+} concentration (Fig. 4*A*) implies that a substantial part of the observed isotonic fiber stiffness may arise from weakly attached cross-bridges. Consequently, occupancy of strong-binding (force-generating) cross-bridge states during high-speed shortening might be substantially less than that previously assumed from stiffness measurements (40% and 15% of the occupancy during isometric contraction for frog and rabbit, respectively) and may then be quite consistent with the small increase in isotonic fiber ATPase over isometric ATPase (42).

For the rising phase of a tetanus, we previously demonstrated (30) that a 5-fold increase in actin affinity of weak-binding cross-bridges with Ca^{2+} can account for the lead of stiffness and equatorial reflections over force development (43–45). The present results further indicate that as Ca^{2+} concentration increases upon activation, the leftward shift of the stiffness–speed relation and the possible presence of some nucleotide-free myosin heads could all enhance the apparent lead of stiffness and equatorial reflections. Some nucleotide-free myosin heads may also account for the appearance of some actin labeling before force generation with features different from the rigor pattern (46). The differences in the rigor pattern may arise from the proposed differences between actomyosin bonds with one nucleotide-free myosin head (incomplete nucleotide saturation) vs. those where both heads are nucleotide free (rigor; refs. 37 and 38).

[§]Titration studies as shown in Fig. 3*A* at pCa 8 with MgATP (and in the presence of a sufficient backup system) indicate full saturation at a MgATP concentration of <20 μM (unpublished results). This concentration is only 5- to 10-fold lower than that for ATP[γS] under the same conditions, suggesting that in the presence of 1–2 mM MgATP at high Ca^{2+} concentrations nucleotide saturation may also be limiting.

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