The Myosin Cross-Bridge Cycle and Its Control by Twitchin Phosphorylation in Catch Muscle

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ABSTRACT The anterior byssus retractor muscle of *Mytilus edulis* was used to characterize the myosin cross-bridge during catch, a state of tonic force maintenance with a very low rate of energy utilization. Addition of MgATP to permeabilized muscles in high force rigor at pCa $>$ 8 results in a rapid loss of some force followed by a very slow rate of relaxation that is characteristic of catch. The fast component is slowed 3–4-fold in the presence of 1 mM MgADP, but the distribution between the fast and slow (catch) components is not dependent on [MgADP]. Phosphorylation of twitchin results in loss of the catch component. Fewer than 4% of the myosin heads have ADP bound in rigor, and the time course (0.2–10 s) of ADP formation following release of ATP from caged ATP is similar whether or not twitchin is phosphorylated. This suggests that MgATP binding to the cross-bridge and subsequent splitting are independent of twitchin phosphorylation, but detachment occurs only if twitchin is phosphorylated. A similar dependence of detachment on twitchin phosphorylation is seen with AMP-PNP and ATPγS. Single turnover experiments on bound ADP suggest an increase in the rate of release of ADP from the cross-bridge when catch is released by phosphorylation of twitchin. Low [Ca^{2+}] and unphosphorylated twitchin appear to cause catch by 1) markedly slowing ADP release from attached cross-bridges and 2) preventing detachment following ATP binding to the rigor cross-bridge.

INTRODUCTION

Some smooth muscles show tonic force maintenance associated with very low rates of energy utilization and very slow shortening velocities. In vertebrate smooth muscles this state of high force output with slow myosin cross-bridge cycling is referred to as "latch" (Dillon et al., 1981), but the most extreme example of this type of mechanical behavior is the "catch" state first identified in the early 1900s in invertebrate smooth muscles (for review see Bayliss, 1927). The catch state is characterized by high force maintenance with very little suprabasal energy usage (Baguet and Gillis, 1968; Minihan and Davies, 1966) and an inability of the muscle to actively redevelop force following a quick release (Jewell, 1959). Cholinergic stimulation of a catch muscle such as the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* results in a rapid increase in force and active shortening if the muscle is released. With time, these properties of the muscle change to the catch state, in which force persists for long periods even though the excitatory stimulus is removed (Jewell, 1959). Serotonergic nerve stimulation results in rapid relaxation of catch force (see Twarog, 1967).

Activation of cross-bridge cycling in molluscan catch muscles results from direct calcium binding to myosin (for review see Szent-Gyorgyi, 1996; Szent-Gyorgyi et al., 1999). When the muscle is stimulated, intracellular [Ca^{2+}] increases and force is generated. [Ca^{2+}] then falls with time, and catch force ensues even though intracellular [Ca^{2+}] approaches resting concentration (Ishii et al., 1989). Relaxation of catch force produced by serotonin is not associated with a change in intracellular [Ca^{2+}], but rather results from an increase in [cAMP] and activation of protein kinase A (Cole and Twarog, 1972; for review, see Twarog, 1976).

The fundamental characteristics of catch can be reproduced in permeabilized ABRM (Cornelius, 1980, 1982; Pfitzer and Ruegg, 1982; Castellani and Cohen, 1987; Siegman et al., 1997). An increase in [Ca^{2+}] causes activation of the muscle and fast cross-bridge cycling as evidenced by a high ATPase rate and rapid shortening. The subsequent return to a "resting" [Ca^{2+}] results in a slowly decaying force with very low ATPase and absence of force redevelopment following a quick release. The catch force relaxes rapidly with addition of cAMP (Butler et al., 1998). We have recently reported that the release of catch force is due to a protein kinase A-mediated phosphorylation of twitchin (Siegman et al., 1997, 1998), a mini-titin that is associated with the myosin-containing filament in catch muscles (Vibert et al., 1993).

The mechanisms by which myosin cross-bridges make the transition from force maintenance with rapid cycling to catch force maintenance with slow cycling, and the mechanism of control of relaxation of catch by the phosphorylation of twitchin, are not yet known. In vertebrate smooth muscle, the latch state has been proposed to arise from the formation of slowly detaching cross-bridges that result from dephosphorylation of the myosin regulatory light chain while the cross-bridge is in the high force state (Hai and

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Abbreviations used: A, actin; AM, actomyosin; AMADP, actomyosin-ADP; AMATP, actomyosin-ATP; M, myosin.

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Murphy, 1988). The accumulation of dephosphorylated, slowly detaching cross-bridges would result in high force maintenance with low cross-bridge cycling rates. Other mechanisms have also been proposed to result in force generation from unphosphorylated cross-bridges with slow cycling rates (Himpens et al., 1988; Vyas et al., 1992; Malmqvist et al., 1997; Haeberle, 1999).

Unphosphorylated, attached cross-bridges in tonic mammalian smooth muscle have a very slow rate of release of ADP (Khromov et al., 1995). Furthermore, such cross-bridges have a very high affinity for MgADP (Fuglsang et al., 1993), with the result that relatively small concentrations of MgADP (~100–150 μM) can effectively compete with MgATP (2 mM) for binding to the rigor cross-bridge (Khromov et al., 1995, 1996, 1998). These findings suggest that latch represents an unphosphorylated cross-bridge with ADP bound. Detachment of the cross-bridge would be limited by the slow rate at which ADP comes off and the competition between ADP and ATP binding to the rigor cross-bridge (Khromov et al., 1995). Because the catch cross-bridge represents an extreme of slow cross-bridge cycling, it was of interest to determine whether cross-bridge detachment in catch was limited by a very slow rate of ADP release, and whether ADP affinity of the rigor cross-bridge in catch might exceed that of tonic mammalian smooth muscle. In addition, the rapid relaxation of catch force by phosphorylation of twitchin suggests that these cross-bridge parameters are likely to be highly regulated.

METHODS

Muscle preparation

Mytilus edulis were obtained from Anastasi's Fish Market, Philadelphia, PA. Mussels were housed in an aquarium containing aerated filtered seawater (Instant Ocean, Carolina Biological Supply, Burlington, NC) at 5°C. On the day of the experiment, the shell was opened, the anterior byssus retractor muscle (ABRM) was exposed, and the pedal ganglia removed. Muscle bundles (0.2–0.4 mm in diameter and up to 1 cm in length) were mounted on holders and incubated in an artificial seawater solution at 20°C until use. The artificial seawater (ASW) contained KCl, 10 mM; $MgCl_2$, 50 mM; $CaCl_2$, 10 mM; NaCl, 428 mM; *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 10 mM at pH 7.4.

Solutions for permeabilized muscles

The compositions of the various solutions are listed in Table 1. A computer program provided by Dr. R. J. Barsotti was used to solve the multiple binding equilibria (see Dantzig et al., 1999). The $[Ca^{2+}]$ of the solutions containing EGTA and no added calcium was considered to be pCa > 8. All experiments were performed at 20°C.

Muscles were permeabilized by incubation for 30 min in a low EGTA (pCa > 8) rigor solution containing 1% Triton X-100. This was followed by at least three washes of 5 min duration in similar rigor solutions without Triton X-100.

Mechanical measurements

Muscle bundles of ~5 mm in length were mounted on a myograph similar to that described previously (Siegman et al., 1984, 1997). Force output was measured with a DSC-6 transducer (Kistler Morse, Bothell, WA) and was recorded on both a strip chart recorder and a digital storage oscilloscope (model 4094 or 310, Nicolet, Madison, WI).

Caged ATP experiments

The flash-lamp apparatus for photolysis of caged ATP and the pneumatically driven freeze clamp device used for rapid freezing of muscles at times <10 s after the flash have been described (Vyas et al., 1994). Caged ATP was obtained from Calbiochem (La Jolla, CA) and was treated with apyrase (Grade V, Sigma Chemical Co., St. Louis, MO) before use. Apyrase was removed by centrifugation through a 5000 D cutoff filter. ADP and ATP were not detectable by high performance liquid chromatography (HPLC) analysis after this treatment. Caged ^{3}H-ATP was synthesized and purified

TABLE 1 Composition of solutions

Solution	Nucleotide	Mg-Nucleotide	CaEGTA	EGTA	HDTA	$MgCl_2$	Mg^{2+}	PCr	Other Constituents
Rigor, low EGTA, pCa > 8	—	—	—	2	42.3	3.6	3	—	
Rigor, pCa > 8	—	—	—	20	22.9	4.7	3	—	
Rigor, pCa 5	—	—	18.1	1.9	23.1	3.7	3	—	
Relaxing, pCa > 8	1.03 ATP	1.0	—	20	21.2	5.6	3	—	
Relaxing, pCa > 8 + PCr	1.03 ATP	1.0	—	20	1.3	7.6	3	20	CPK, 1 mg/ml
Activating, pCa 5	1.03 ATP	1.0	18.1	1.9	21.4	4.7	3	—	
Activating, pCa 5 + PCr	1.03 ATP	1.0	18.1	1.9	1.5	6.6	3	20	CPK, 1 mg/ml
ATPγS, pCa > 8	8.24 ATPγS	8.0	—	20	9.1	12.5	3	—	0.5 AP_5A
AMP-PNP, pCa > 8	5.6 AMP-PNP	5.4	—	20	13.6	9.4	3	—	
ATP + ADP, pCa > 8	1.03 ATP, 1.52 ADP	1.0 ATP 1.0 ADP	—	20	19.5	6.6	3	—	
Caged ATP, pCa > 8	1.0 caged ATP	0.46	—	20	21.4	5.1	3	—	0.5 AP_5A
Caged ATP, pCa > 8	4.0 caged ATP	2.0	—	20	16.4	7.3	3.5	—	0.5 AP_5A
Caged ATP + ADP, pCa > 8	4.0 caged ATP, 1.52 ADP	2.0 cgATP, 1.0 ADP	—	20	14.8	8.3	3.5	—	0.5 AP_5A

All concentrations are mM, and all solutions contained the following: 30 mM piperazine-*N*,*N*′-bis[ethanesulfonic acid] (PIPES), 5 mM Pi, 0.5 mM leupeptin, and 1 mM dithiothreitol. Ionic strength was 202 mM and pH was 6.8. HDTA, 1.6-diaminohexame-*N*,*N*,*N*′,*N*′-tetraacetic acid; PCr, phosphocreatine; CPK, creatine phosphokinase; AP_5A, P1,p5-di(adenosine-5′)pentaphosphate; ATPγS, adenosine 5′-*O*-(γ-thiotriphosphate, AMP-PNP, 5′-adenylylimidodiphosphate.

as described previously (Vyas et al., 1994). The desired specific activity of the caged ^{3}H-ATP used for individual experiments was obtained by mixing unlabeled caged ATP with the purified caged ^{3}H-ATP having a specific activity of ~8 Ci/mmol. Because caged ATP has a lower affinity for Mg^{2+} than ATP, the free Mg^{2+} of the unphotolyzed solution was adjusted as shown in Table 1.

Muscle freezing and extraction of nucleotides

Muscles were routinely frozen by immersion in liquid N_2 except in experiments in which the time course of ADP formation was determined following photolysis of caged ^{3}H-ATP. Muscles were pulverized in frozen 0.5 N $HClO_4$, and the acid extract adjusted to pH 7.4 with KOH. The extract was subjected to HPLC on a versapak NH_2 column (Alltech Associates, Deerfield, IL) using mobile phases of 0.05–0.5 M $NH_4H_2PO_4$, 5 mM EDTA, pH 4.0. The [$NH_4H_2PO_4$] and the mobile phase gradients used for a particular experiment were adjusted to give a good separation of the nucleotides of interest. The column effluent was collected, scintillation fluid added, and radioactivity determined in a liquid scintillation counter. A similar separation technique was used to monitor the purity of the various nucleotides added to the solutions for the permeabilized muscles.

Phosphorylation and thiophosphorylation of twitchin

Twitchin was phosphorylated in the permeabilized muscles by the addition of cAMP (100 μM) in the presence of MgATP (Siegman et al., 1997; Butler et al., 1998). In some experiments, thiophosphorylation of twitchin was accomplished by incubation of the muscle in a rigor solution containing ATPγS (100 μM) and cAMP (100 μM). This was followed by several washes of the muscle in rigor solution. Previous experiments have shown that twitchin is thiophosphorylated by this procedure, and that this thiophosphorylation is resistant to phosphatase activity (Siegman et al., 1997, 1998). Muscles treated in this way were then subjected to the protocols of interest and are designated as "twitchin prethiophosphorylated."

Other materials

[2,8-^{3}H] Adenosine 5′-triphosphate (35 Ci/mmol) and [8-^{14}C] adenosine 5′-triphosphate (56 Ci/mol) were obtained from NEN Life Science (Boston, MA) and [U-^{14}C] D-mannitol (32 Ci/mol) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA).

ATPγS was obtained from Calbiochem and was treated overnight at room temperature with apyrase (1 mg/ml) in a solution containing 30 mM PIPES, 100 mM $MgCl_2$, and 100 mM ATPγS, pH 6.85. This was followed by filtration of the solution through a 5000 Da cutoff filter. The procedure resulted in a decrease in ADP from ~10% to <0.5% of the ATPγS, with only a small loss of ATPγS. After treatment, ~10% of the total absorbance at 259 nm was in AMP and nucleosides.

AMP-PNP (lithium salt, Sigma) was purified by chromatography on an Econosil NH_2 column (Alltech Associates) using a modification of a previously described method (Axelson et al., 1981). The AMP-PNP initially contained ~8% AMP-PN, and after the purification procedure this was reduced to <0.8%.

Statistics

Data are expressed as mean ± SEM. Statistical analyses were performed using either the *t*-test or one-way ANOVA.

RESULTS

Fig. 1 *A* shows the protocol for the production of catch force in the permeabilized ABRM. When the muscle is transferred to a relaxing solution after activation in pCa 5, there is an initial rapid relaxation followed by a much slower rate of decrease in force. We have previously shown that the force maintained in pCa > 8 is associated with a very low ATPase and a loss of force redevelopment following a rapid decrease in muscle length. The addition of cAMP causes the phosphorylation of twitchin and a rapid decrease of catch force. Also, if twitchin is prethiophosphorylated by treatment of the muscle with ATPγS and cAMP, catch is prevented and transfer to low [Ca^{2+}] results in rapid relaxation (Siegman et al., 1997).

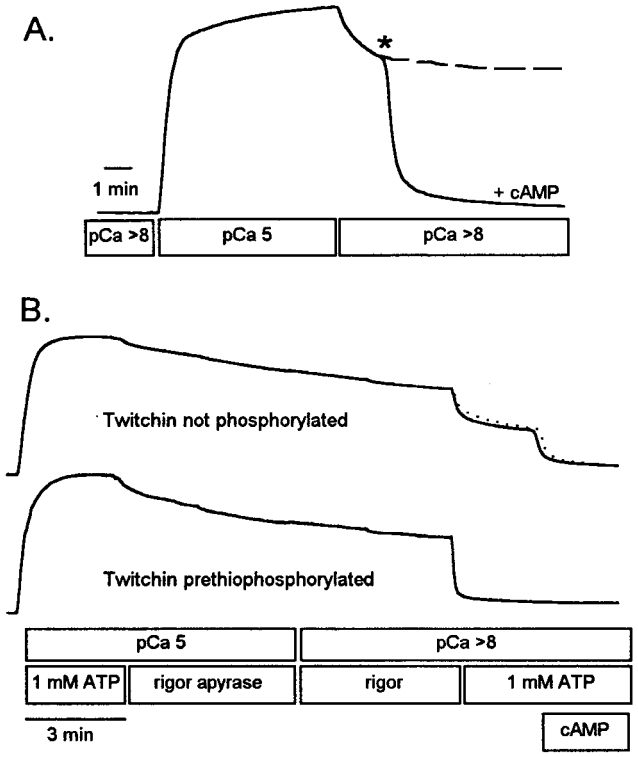


FIGURE 1 Force responses of the permeabilized ABRM. (*A*) After activation in pCa 5, transfer of the muscle to pCa > 8 results in a decrease in force followed by catch force maintenance (*dashed line*). Addition of cAMP (0.1 mM) at * (*solid line*) relaxes catch force. (*B*) Force responses when ATP is added to ABRM in high force rigor. Muscles were activated in pCa 5, and then transferred to a pCa 5 rigor solution containing apyrase (0.2 mg/ml). [Ca^{2+}] was then lowered to pCa > 8. The solid lines shows the response to addition of 1 mM MgATP and subsequently cAMP. In the lower trace, the muscle was pretreated with ATPγS (0.1 mM) and cAMP (0.1 mM) before initial activation of the muscle. This results in irreversible thiophosphorylation of twitchin. The dotted line in the top trace shows the response to addition of a solution containing 1 mM MgATP, 20 mM phosphocreatine and creatine phosphokinase.

Relaxation of rigor force with MgATP

In order to study the cross-bridge cycle in catch muscle, we felt that it was appropriate to start with the cross-bridges in a well-defined state such as rigor. The first experimental

design addressed the question of whether catch cross-bridges in rigor detach following addition of ATP, and whether twitchin phosphorylation plays a role in regulating such detachment. The muscles were put into a high force rigor state by removing ATP during activation in pCa 5. Following treatment with apyrase to minimize [ATP] and [ADP], the muscle was transferred to the low [Ca^{2+}] solution followed by addition of ATP. Typical force responses are shown in Fig. 1 *B*. In muscles in which twitchin was not phosphorylated, addition of ATP resulted in a rapid decrease of ~50% of the rigor force with the remaining force declining at a much slower rate. The subsequent addition of cAMP caused a further reduction of force. In muscles in which twitchin was thiophosphorylated before the rigor treatment, the addition of ATP resulted in the rapid loss of almost all of the rigor force with no subsequent effect of cAMP. These results suggest that when twitchin is not phosphorylated, the addition of ATP to muscles in rigor results in some cross-bridges remaining attached to actin in the catch state.

Role of MgADP

Because the affinity of myosin for MgADP can be very high in some smooth muscles (Fuglsang et al., 1993), we carried out several experiments to test whether the persistence of the catch state after rigor treatment was due to ADP remaining on the cross-bridge in the rigor solution or to ADP competing with ATP for binding to the rigor cross-bridge. Total [ADP] in solutions containing 20 mM phosphocreatine, 1 mM MgATP, and creatine phosphokinase was 0.8 ± 0.2 μM ($n = 6$), making MgADP ~0.5 μM. Addition of this solution containing very low [MgADP] and an ATP regenerating system to the muscle following rigor treatment caused a similar mechanical response (*dotted line*, Fig. 1 *B*). The persistence of catch force is not likely due to ADP competing with ATP for binding to the rigor cross-bridge, unless the myosin in rigor has an extraordinarily high affinity for MgADP.

Another possible mechanism for the persistence of catch force following treatment in rigor solution is that ADP remains bound to the cross-bridge. If so, the catch force after addition of ATP would result from AMADP cross-bridges already present. The addition of ATP would have no effect on force output from these cross-bridges until ADP was released and ATP could subsequently bind. In order to investigate this possibility, we measured the ADP content of the muscle in rigor. The procedure involved activation (pCa 5) of the muscle in a solution containing ^{3}H-ATP so that any ADP bound to myosin would contain tritium. The muscles were then subjected to the rigor protocol and frozen. The results are shown in Table 2. Also included are data from muscles that were frozen in a pCa 5 solution containing ATP and phosphocreatine. The latter represents ADP bound during cross-bridge cycling and force maintenance, and has been shown to be a good estimate of myosin S1 concentration in mammalian smooth muscle (Butler et al., 1989). Both [ADP] and [ATP] were very low in the rigor muscles. Furthermore, there was no significant effect of thiophosphorylation of twitchin on [ADP] (difference = 0.3 ± 0.5 μM). We conclude that only a very small fraction (<4%) of myosin retains ADP following the rigor protocol. It is unlikely that a slow ADP release from such a small fraction of myosin could result in the catch force maintenance seen following the addition of ATP to the rigor muscles. Also, the large dependence of mechanical response on the phosphorylation state of twitchin is not matched by a difference in ADP content in the two groups.

TABLE 2 Nucleotide contents of muscles in rigor

Treatment	*n*	ATP (μM)	ADP (μM)
Rigor, twitchin not phosphorylated	7	1.4 ± 1.0	2.5 ± 0.3
Rigor, twitchin thiophosphorylated	7	0.8 ± 0.6	2.2 ± 0.4
pCa 5*	11	1000*	66 ± 13*

Muscles initially in rigor were incubated in a pCa 5 solution containing ^{3}H-ATP (1 mM, 0.19 mCi/ml) for 3 min and then transferred to pCa 5 rigor solution containing apyrase (0.02 mg/ml) for 5 min. This was followed by incubation in pCa > 8 for an additional 5 min before freezing. All solutions contained ^{14}C-mannitol (1 mM, 17.5 μCi/ml) as a volume marker. The nucleotides were extracted, separated by HPLC and the ^{3}H and ^{14}C dpm used to calculate the concentration of the original labeled nucleotide remaining.

*These muscles were frozen in a solution containing 1 mM MgATP, 20 mM phosphocreatine, and creatine phosphokinase (1 mg/ml). Data are mean ± SEM.

Kinetics of relaxation of rigor force following photolysis of caged ATP

In an effort to further characterize the mechanism by which catch force persists following addition of ATP to a rigor muscle, we performed experiments in which a rapid increase in [MgATP] was initiated by photolysis of caged ATP. Muscles were put into high force rigor using a procedure similar to that shown in Fig. 1 *B*, followed by addition of caged ATP and subsequent flash photolysis. Fig. 2 *A* shows typical force responses following the flash. When twitchin was prethiophosphorylated, the increase in ATP resulted in a rapid fall in force with little or no further decrease upon addition of cAMP. In contrast, when twitchin was not phosphorylated, there was a much smaller rapid decrease in force and a large subsequent relaxation with cAMP treatment. Fig. 2 *B* shows the initial time course of the fall in force normalized to the total decrease in force during the 10 s following the flash. Interestingly, the time course of the fast component is independent of the state of twitchin phosphorylation. A simple interpretation of these data is that there are two types of cross-bridges. One type detaches rapidly, and the other type (representing those in

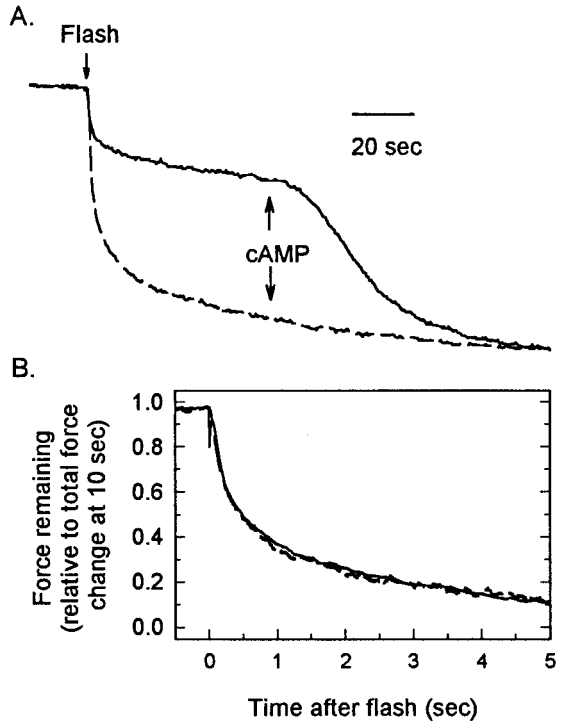


FIGURE 2 Force responses following release of ATP from caged ATP. Muscles were put into high force rigor using a protocol similar to that shown in Fig. 1 *B*. Caged ATP (4 mM) was added 4 min before the flash, and the muscle was transferred to a solution containing 1 mM MgATP ~4 s following the flash. cAMP was added at the time shown. *Solid lines*, twitchin not phosphorylated; *dashed lines*, twitchin thiophosphorylated. (*A*) Force responses normalized to the total force change in the 2.5 min after the flash including the addition of cAMP. (*B*) force responses normalized to the total force change at 10 s following the flash.

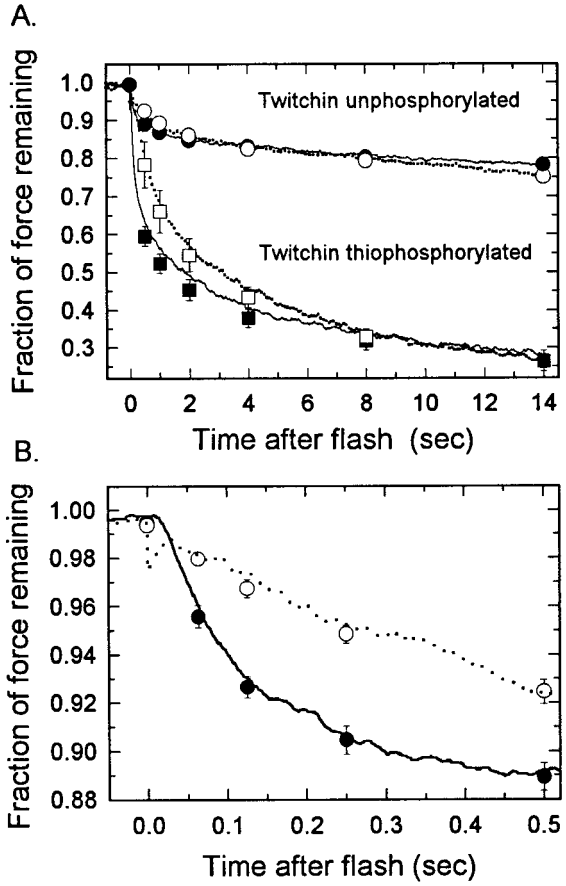


FIGURE 3 Effect of [MgADP] on the force response to photolysis of caged ATP in muscles in high-force rigor. High-force rigor was developed according to the protocol shown in Fig. 1 *B*. MgADP (1 mM) was included in the caged ATP solution; and 15 s after photolysis, the muscle was transferred to a solution containing MgATP (1 mM) and MgADP (1 mM). cAMP (100 μM) was added 2 min later. Control muscles were treated identically except no MgADP was added. The force remaining is shown as a fraction of the total change in force that results from photolysis of caged ATP and subsequent treatment with cAMP in each design. The symbols show the force remaining in the presence (*open symbols*) or absence (*closed symbols*) of 1 mM MgADP when twitchin is thiophosphorylated (*squares*) or not (*circles*). Typical force traces from muscles in the presence (*dotted lines*) or absence (*solid lines*) of MgADP are also shown. *B* shows expanded time scale data from muscles in which twitchin was unphosphorylated. All data points are mean ± SEM, $n = 4$.

catch) detaches very slowly. Twitchin phosphorylation appears to move catch cross-bridges into the fast detaching group.

Effect of MgADP on the kinetics of relaxation of rigor force

The next question that was addressed was whether addition of MgADP could alter the distribution of cross-bridges between the fast and very slowly detaching groups. The experimental design was similar to that used in Fig. 2 except that in some muscles 1 mM MgADP was included in the caged ATP solution. Fig. 3 *A* shows the force remaining as a fraction of the total change in force that results from addition of ATP and subsequent treatment with cAMP. In the absence of ADP, the half-time for the decrease in force that occurred within 14 s after the flash was similar whether twitchin was phosphorylated or not (0.37 ± 0.05 s and 0.44 ± 0.05 s, respectively, $n = 4$ in each). However, the fraction of force in the fast component was much larger when twitchin was thiophosphorylated. In the presence of 1 mM MgADP, there is a slower rate of decrease in force following photolysis of caged ATP. The half-time increased to 1.39 ± 0.18 s and 1.24 ± 0.29 s ($n = 4$ in each) in the absence and presence of twitchin thiophosphorylation, respectively. The dramatic effect of MgADP on the early time course (500 ms following photolysis) is shown in Fig. 3 *B* for muscles in which twitchin was unphosphorylated. In contrast, the force remaining after 2 s is not significantly different in the presence or absence of 1 mM MgADP (see Fig. 3 *A*). When twitchin is unphosphorylated, it appears that the effect of MgADP is to slow the rapidly detaching cross-bridges, but these cross-bridges still detach much more rapidly than those exhibiting catch. The entire effect

of a high concentration of ADP is seen on the fast detaching cross-bridges and is complete in the first several seconds. These results show that the addition of MgATP in the presence of a high concentration of MgADP does not result in a conversion of rapidly detaching cross-bridges into catch cross-bridges that would detach very slowly over a period of several minutes.

Does the rigor catch cross-bridge bind and split MgATP?

The observations that addition of ATP to a rigor muscle does not relax catch force and that the amount of catch force that persists following rigor is independent of the [MgADP] raise the possibility that the catch cross-bridge is an attached rigor state that binds neither ATP nor ADP. When ATP is liberated from caged ATP, both skeletal and cardiac muscles in rigor show a rapid burst of ADP formation that is approximately equal to the myosin S1 concentration under both relaxing and activating conditions (Ferenczi et al., 1984; Barsotti and Ferenczi, 1988). Since the burst of ADP is thought to result from the splitting of ATP on myosin, it should not occur if the rigor cross-bridge does not bind ATP. It should then be possible to detect a decrease in the magnitude of the ADP burst if the rigor cross-bridge in catch does not bind ATP. To test this, we measured the time course of ^{3}H-ADP formation following the photolysis of caged ^{3}H-ATP in rigor muscles whether or not twitchin was prethiophosphorylated. The protocol for these experiments was similar to that shown in Fig. 2 except that radiolabeled caged ATP was used, and the muscles were frozen 0.2 to 10 s after the flash. The results are shown in Fig. 4. There is a rapid burst of ADP formation that is substantially complete by the first measurement at 200 ms. This is followed by a much slower rate of ADP formation. The magnitude of the ADP burst is similar to the magnitude of the ^{3}H-ADP bound in muscles that were frozen in the steady state in a pCa 5 solution containing ^{3}H-ATP and phosphocreatine. As described earlier, this has been shown to be a good estimate of myosin S1 concentration in muscle, and the ADP burst in these experiments is thus approximately equal to the myosin S1 concentration. Importantly, there is no effect of twitchin thiophosphorylation on the time course of ADP formation, even though there is a large effect of such a thiophosphorylation on the mechanical response following photolytic release of ATP (see Figs. 2 and 3). Therefore, we conclude that there is similar ATP binding to myosin and rapid splitting to bound products, whether there is rapid relaxation when twitchin is thiophosphorylated or whether there is persistence of catch force when twitchin is unphosphorylated. These results suggest that unphosphorylated twitchin prevents the detachment of the rigor catch cross-bridge following ATP binding and that it traps the very slowly cycling catch cross-bridge in an ADP-bound state.

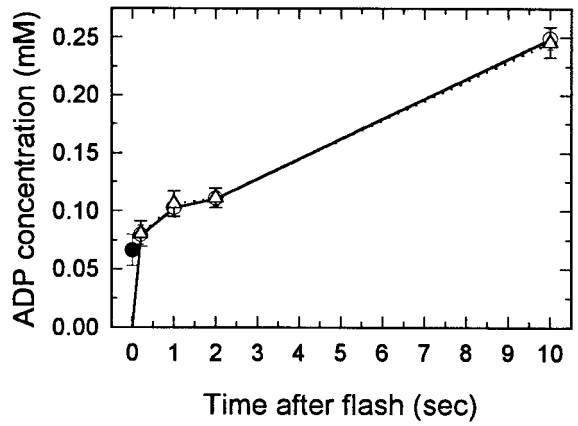


FIGURE 4 Time course of ADP formation following release of ATP from caged ATP. The muscles were put into a high-force rigor according to the protocol shown in Fig. 1 *B*, ending with the muscles in pCa > 8. This was followed by incubation for 4 min in 4 mM caged ATP containing caged ^{3}H-ATP (~0.5 mCi/ml), pCa > 8. The muscles were then subjected to flash photolysis and frozen 0.2 to 10 s later. The muscles were extracted, nucleotides separated on HPLC, and the dpm in appropriate fractions used to calculate the concentration of ADP formed at various times. The muscles were either pretreated with cAMP and ATPγS to thiophosphorylate twitchin (*triangles*, *dotted line*) or not (*open circles*, *solid line*). Also shown at zero time (*solid circle*) is the ^{3}H-ADP present in muscles frozen following incubation in a pCa 5 solution containing ^{3}H-ATP, phosphocreatine (20 mM), and creatine phosphokinase (1 mg/ml). Data are mean ± SEM. n = 3–5 for each time course point and n = 11 for the ^{3}H-ADP present in pCa 5.

Effect of ATP analogs on relaxation of rigor force

In order to further probe the mechanism responsible for the persistence of catch force maintenance after rigor, we used the ATP analogs AMP-PNP and ATPγS. These analogs are non- or slowly hydrolyzable and result in weak binding cross-bridge states when bound to myosin (Kraft et al., 1992; Frisbie et al., 1998). In these experiments, muscles were put into a high force rigor followed by the addition of MgAMP-PNP (Fig. 5 *A*) or MgATPγS (Fig. 5 *B*) at pCa > 8. When twitchin was not phosphorylated, the addition of MgAMP-PNP resulted in a relatively small, rapid fall in force followed by a slower rate of relaxation. When twitchin was prethiophosphorylated, the initial rapid fall in force was much larger (Fig. 5 *A*). The mechanical responses to addition of MgATPγS showed a similar dependence on twitchin phosphorylation (Fig. 5 *B*). In the case of MgATPγS it was also possible to directly show the effect of twitchin phosphorylation by adding cAMP during the slow fall in catch force. When twitchin is thiophosphorylated, there is a large increase in rate of decline in force (Fig. 5 *B*). These results for both AMP-PNP and ATPγS are similar to those obtained for the addition of MgATP (see Fig. 1 *B*). This suggests that the persistence of the catch state following rigor does not require splitting of nucleotide on myosin, and that the effects of twitchin phosphorylation on force output

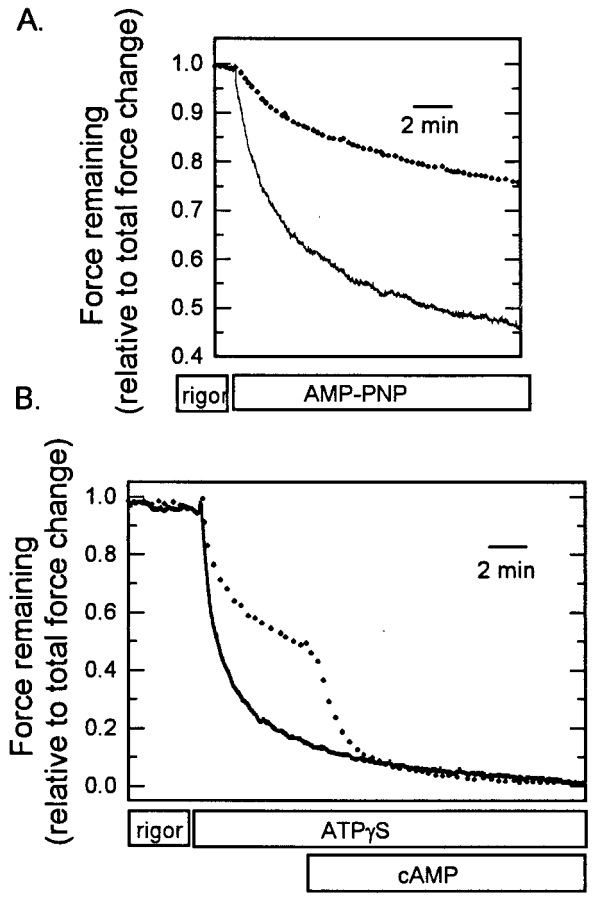


FIGURE 5 Force decrease from rigor induced by AMP-PNP and ATPγS. In (*A*) and (*B*), muscles were put into a high-force rigor according to the protocol shown in Fig. 1 *B*. (*A*) Typical force responses are shown for addition of MgAMP-PNP (5.4 mM) to rigor muscles in which twitchin was prethiophosphorylated (*solid line*) or not phosphorylated (*dotted line*). The force remaining is shown as a fraction of the total decrease in force that occurred following the addition of AMP-PNP and subsequent addition of 1 mM MgATP and cAMP (100 μM) at the end of the experiment. (*B*) Force responses are shown for addition of MgATPγS (8 mM) to rigor muscles in which twitchin was prethiophosphorylated (*solid line*) or not phosphorylated (*dotted line*). cAMP was added to both muscles at the time shown in the figure. Apyrase was included in all solutions following development of high-force rigor. The force remaining is normalized to the total change in force from rigor to that at the end of the experiment in a solution containing MgATPγS and cAMP.

extend to the regulation of the detachment rate of the cross-bridge with nucleoside triphosphate bound.

The results described so far suggest that at low [Ca^{2+}] unphosphorylated twitchin has a major effect on at least two steps of the cross-bridge cycle. Twitchin prevents the detachment of the rigor catch cross-bridge when it binds MgATP. In addition, it appears to trap the cross-bridge in an ADP-bound force-maintaining state. The next series of experiments was designed to investigate the turnover of myosin-bound ADP in the catch state and when catch is released by the cAMP-mediated phosphorylation of twitchin.

Turnover of myosin-bound ADP during catch force maintenance

In order to determine the turnover of myosin-bound ADP in the catch state, muscles were put into high force rigor and subjected to flash photolysis of caged ^{3}H-ATP, as shown by the design in Fig. 6 *A*. As shown earlier, this results in tritium-labeled ADP being bound to myosin both when twitchin is unphosphorylated and when twitchin is prethiophosphorylated. ^{14}C-ATP containing 20 mM phosphocreatine was added 7 s after the flash and the muscles were frozen 2 min later. In the case when twitchin is unphosphorylated, there is catch force maintenance during the time the muscle is in ^{14}C-ATP, but when twitchin is prethiophosphorylated, catch force is relaxed before ^{14}C-ATP is added. As expected, there was no significant difference in the total exchangeable ADP (the sum of ^{3}H-ADP and ^{14}C-ADP) in the two designs (radiolabeled ADP = 62 $\pm$ 3 μM and 61 $\pm$ 9 μM for prethiophosphorylated and unphosphorylated twitchin, respectively). However, in muscles maintaining catch force because twitchin was unphosphorylated, ^{14}C-ADP was a significantly higher fraction of the total ADP (0.325 $\pm$ 0.010 versus 0.242 $\pm$ 0.016, for unphosphorylated and thiophosphorylated twitchin, respectively, n = 4 in each). This higher turnover of bound ADP in muscles maintaining catch force suggests that cross-bridges that maintain catch force do indeed cycle and utilize ATP at a rate faster than cross-bridges in muscles in which catch force is not maintained. It is important to note, however, that the extra turnover of bound ADP associated with catch force maintenance is small and is measured over a long time period (2 min). Therefore, it represents a very slow rate of ATPase during catch.

Turnover of myosin-bound ADP during relaxation of catch

The next experiment was designed to determine whether the *release* of catch force maintenance by cAMP-mediated phosphorylation of twitchin is associated with an increase in the turnover of myosin-bound ADP. This would be expected if the primary cross-bridge state has ADP bound in catch and if the relaxation caused by phosphorylation of twitchin was associated with the release of ADP followed by ATP binding and splitting on myosin. The design is illustrated in Fig. 6 *B*. Twitchin was not prethiophosphorylated in either design. At 7 s following flash photolysis of caged ^{3}H-ATP, the muscles were placed in a solution containing ^{14}C-ATP and phosphocreatine (20 mM) for 15 s; cAMP was then added to one of the muscles (*top trace*) which was frozen 30 s later. The control muscle (*bottom trace*) was treated identically except that cAMP was not added. In paired comparisons, there was a 16 $\pm$ 6% (n = 8, $p < 0.05$) higher ratio of ^{14}C: ^{3}H in ADP in the muscles that have relaxed from catch because of the treatment with

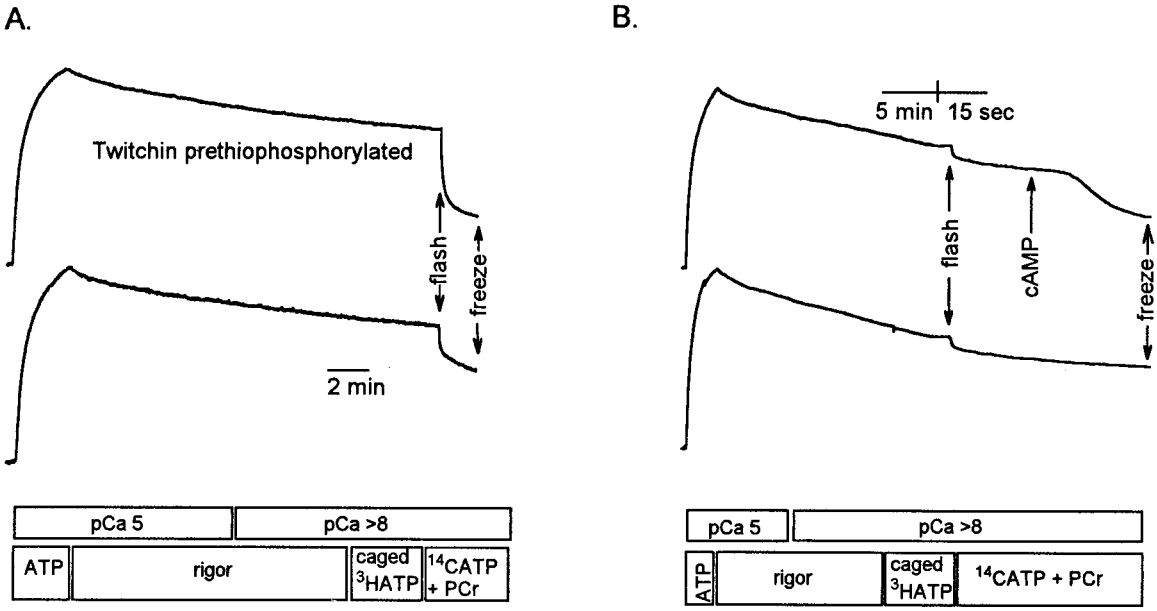


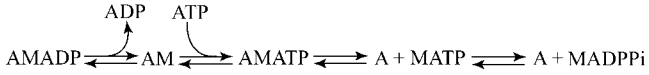
FIGURE 6 Protocols and typical force traces in experiments used for determination of the turnover of bound ADP during catch force maintenance and during relaxation of catch force. (*A*) Protocol for measurement of the turnover of myosin-bound ADP in the catch state. Muscles in high-force rigor were incubated in 1 mM caged ATP containing caged ^{3}H-ATP (1.25 mCi/ml), pCa $>$ 8. At 7 s following the flash, the muscles were placed in a solution containing 1 mM MgATP (^{14}C-ATP, 56 μCi/ml) and phosphocreatine (20 mM) for 2 min. Muscles were frozen, nucleotides extracted, and ^{14}C and ^{3}H dpm in ADP and ATP determined. All solutions from 5 min before addition of caged ATP to the end of the experiment contained creatine phosphokinase. Muscles were either were treated with ATPγS and cAMP before initiation of the experiment to cause irreversible thiophosphorylation of twitchin (*top trace*) or twitchin was not phosphorylated (*bottom trace*). (*B*) Protocol for determination of the turnover of bound ADP during cAMP-mediated relaxation of catch force. The protocol was similar to that shown in (*A*) until the flash, except that twitchin was not prethiophosphorylated in either experimental design. At 7 s following the flash, the muscles were placed in a solution containing 1 mM MgATP (^{14}C-ATP, 56 μCi/ml) and phosphocreatine (20 mM) for 15 s. cAMP was then added in one of the designs (*top trace*) and the muscle frozen 30 s later. The other design (*bottom trace*) was identical except that cAMP was not added.

cAMP. These results are consistent with the idea that detachment of catch force maintaining cross-bridges is associated with release of ADP from myosin and subsequent binding and splitting of ATP.

DISCUSSION

Addition of ATP to the ABRM in high force rigor at pCa $>$ 8 results in a rapid loss of less than one-half of the force over a time period of several seconds, with the remaining force showing a very slow rate of decay over many minutes. The characteristics of the fast component of force decay are similar to those expected from muscles that do not display catch. The overall time course of relaxation of this component following photolysis of caged ATP (Figs. 2 and 3) is similar to that reported for apyrase-treated rabbit femoral artery (Fuglsang et al., 1993). Our interpretation is that the cross-bridges contributing to this initial rapid relaxation represent a subset of cross-bridges that do not participate in catch force maintenance under the conditions studied. The long-lasting force that persists after addition of ATP is due to catch cross-bridges, which rapidly detach when cAMP is added and twitchin is phosphorylated. The premise that maintenance of catch force results from force-bearing links between myosin and actin raises the question as to how the transition from the rigor state to the catch state in the presence of ATP occurs without apparent detachment of these cross-bridges and loss of force.

Experiments probing the maintenance of tonic force in mammalian smooth muscle have been analyzed using Scheme 1 (Khromov et al., 1995, 1996).



In such a scheme, the long-term persistence of force following addition of ATP to a muscle subjected to rigor treatment could result if 1) a significant fraction of the myosin in the rigor muscle still has ADP bound, and/or 2) there is binding of ADP rather than ATP to the rigor cross-bridge. In the first case, the cross-bridge would not detach until ADP was released, and this could be a very slow process in catch. In the second case, relaxation would be delayed because AMADP is reformed with ADP binding. Both have been shown to contribute to slow detachment of cross-bridges in mammalian smooth muscle (Fuglsang et

al., 1993; Khromov et al., 1995, 1996, 1998). The results from the experiments reported here do not support either of these possibilities for the persistence of catch force following addition of ATP to a muscle subjected to a rigor protocol. Measurements of [ADP] show that only a very small fraction of total myosin could have ADP bound in the apyrase-treated muscles (Table 2). Also, there is no apparent difference in the amount of catch force maintained when ATP is added at [MgADP] varying from <1 μM to 1 mM (see Figs. 1 and 3).

Even though there is a lack of an effect of [MgADP] on the magnitude of catch force following addition of ATP, there was a slowing in the rapid decrease in force in the initial seconds following addition of ATP by photolysis of caged ATP (Fig. 3). The effects of MgADP shown here are similar to those reported by Galler and colleagues (1999) who found that in the ABRM, the force decay following release of ATP was slowed ~5-fold in the presence of 0.5 mM MgADP. They also note that even in the presence of high [MgADP], the decrease in force is too rapid to account for catch. The addition of high concentrations of [MgADP] to the rigor muscle is *not* sufficient to convert the otherwise rapidly detaching cross-bridges to catch cross-bridges. The effect of MgADP on this rapidly detaching subset of cross-bridges is as expected using the analysis shown in Scheme 1, and is broadly similar to that seen in experiments on a variety of smooth (Fuglsang et al., 1993), skeletal (Dantzig et al., 1991), and cardiac (Martin and Barsotti, 1994) muscles. An important finding in the ABRM is that no manipulation involving [MgADP] and/or the ratio [MgATP]:[MgADP] changed the fraction of cross-bridges that comprises this rapidly detaching subset. It is the distribution of cross-bridges into those that detach rapidly after the addition of ATP and those that detach with a much slower rate that is controlled only by the phosphorylation state of twitchin.

We also considered the possibility that catch force maintenance after addition of ATP could result from catch being a rigor state (AM) which for some reason binds neither ADP nor ATP. This does not appear to be the case, since the magnitude of the rapid burst of ADP formation following the photolytic release of ATP from caged ATP is similar to the bound ADP present in a contracting muscle at pCa 5 (see Fig. 4). Rather, it appears that most of the myosin binds ATP and splits it to ADP and Pi, whether or not twitchin is phosphorylated. If twitchin is phosphorylated, the cross-bridge detaches; if twitchin is not phosphorylated, detachment of the catch cross-bridge does not occur.

The experiments using analogs of ATP (Fig. 5) further delineate the steps in the cross-bridge cycle that are controlled by phosphorylation of twitchin. The effects of twitchin phosphorylation on the mechanical responses to additions of AMP-PNP and ATPγS to muscles in high-force rigor are very similar to those seen with ATP. The magnitude of the rapid decrease in force following addition of nucleoside triphosphate is much larger when twitchin is thiophosphorylated. X-ray diffraction studies (Frisbie et al., 1998) and mechanical studies (Heizmann et al., 1997) have shown that the complex of AMP-PNP with myosin is similar to the weak binding cross-bridge state normally present in relaxed muscles. Similar results have been reported for the slowly hydrolyzable analog MgATPγS (Kraft et al., 1992). Our findings of continued maintenance of catch force in the presence of these analogs of ATP suggest that when the catch cross-bridge binds the analog it does not detach from actin. That is, the catch cross-bridge with nucleoside triphosphate bound does not detach from actin until twitchin is phosphorylated. Detachment of the catch cross-bridge is very slow whether ADP or nucleoside triphosphate is bound. Therefore, in the absence of calcium and twitchin phosphorylation, a cross-bridge transition necessary for detachment is prevented whether myosin has nucleoside diphosphate or triphosphate bound.

The persistence of catch force maintenance following rigor in the presence of the non-hydrolyzable analog AMP-PNP argues against the idea that maintenance of catch force after rigor could result from the cooperative reattachment of cross-bridges that have initially detached from actin. Such a reattachment would have to occur without the splitting of AMP-PNP, and it is unlikely that the cross-bridge could make the transition from the weakly bound to the strongly bound state without such splitting.

These data strongly suggest that the catch cross-bridge in rigor binds MgATP, but does not detach from actin. This inhibition of the detachment of myosin following binding of MgATP to the rigor cross-bridge obviously facilitates the long-term maintenance of force in the catch state. Indeed, no matter what other steps in the cross-bridge cycle are regulated in catch, inhibition of detachment is sufficient to result in catch force maintenance.

We have further investigated the control of the cross-bridge cycle in the catch muscle by using single turnover experiments to characterize the turnover of myosin bound ADP. The results in the catch muscle show an extra turnover of bound ADP when 1) the muscle maintains catch force compared to when catch force is not present, and 2) catch force is relaxed by addition of cAMP and phosphorylation of twitchin.

Although the turnover of bound ADP during catch force maintenance was higher than in the absence of catch force, the rate is quite slow. With the simple assumption that all myosin cycles at the same rate, the rate constant for ADP turnover during catch would be 0.2 min^{-1}. This is almost 200-fold slower than the ATPase rate of 0.6 s^{-1} measured during maximal activation of the permeabilized ABRM (Butler et al., 1998). There have been other reports of small, but measurable, suprabasal ATPases associated with catch force maintenance in both intact (Baguet and Gillis, 1968) and skinned (Butler et al., 1998) ABRM.

The observation that the rigor catch cross-bridge appears to rapidly bind ATP and split it to ADP and Pi (Fig. 4), together with the fact that the ATPase during catch is very slow, suggests that the primary cross-bridge state during catch is one with ADP bound to myosin. A likely scenario for the release of catch following phosphorylation of twitchin would be the release of ADP from the actin-bound cross-bridge followed by ATP binding, detachment of the cross-bridge, and subsequent splitting of ATP on detached myosin. This is essentially the completion of a "normal" cross-bridge cycle. The single turnover experiments reported here show an extra turnover of bound ADP when catch force is released by addition of cAMP and phosphorylation of twitchin. This supports the idea that phosphorylation of twitchin facilitates the release of ADP from the catch cross-bridge. At low [Ca^{2+}], the cross-bridge appears to be "trapped" in an attached, ADP-bound state when twitchin is unphosphorylated. Phosphorylation of twitchin allows the cross-bridge to proceed to the release of ADP and eventual detachment of the cross-bridge.

In summary, the essential elements of the cross-bridge cycle in catch and its regulation by twitchin phosphorylation are twofold. The first is that the cross-bridge in catch is an ADP bound state that releases ADP very slowly. This must result from a dramatic slowing of at least one reaction preceding the release of ADP from actin-bound myosin. This accounts for the very slow ATPase associated with catch and keeps the cross-bridge attached to actin. The second aspect of the catch cross-bridge is inhibition of the detachment of myosin from actin following binding of MgATP to the rigor cross-bridge. This keeps the cross-bridge attached to actin in catch even if ADP is released from AMADP. Both ADP release from AMADP and cross-bridge detachment following binding of MgATP are increased by phosphorylation of twitchin.

A proposal for the mechanism of catch

Fig. 7 shows a cross-bridge cycle that is consistent with the results from experiments described here. Regulation of the catch state in this cycle is based mainly on the postulate that the unbinding of calcium from an attached force generating cross-bridge traps the cross-bridge in a force-generating state in the absence of twitchin phosphorylation, but not when twitchin is phosphorylated. It is an extension of a model that we have previously described (Butler et al., 1998).

It is assumed that the myosin in ABRM exhibits two force-generating states, as has been described for vertebrate smooth muscle and non-muscle myosins (Whittaker et al., 1995; Jontes et al., 1995; Gollub et al., 1996; Jontes and Milligan, 1997). The first state is designated M_1 and the second, which is characterized by an extra swing of the lever arm of myosin, is designated M_2 in Fig. 7. We propose that the rate of isomerization of myosin from M_1 to M_2

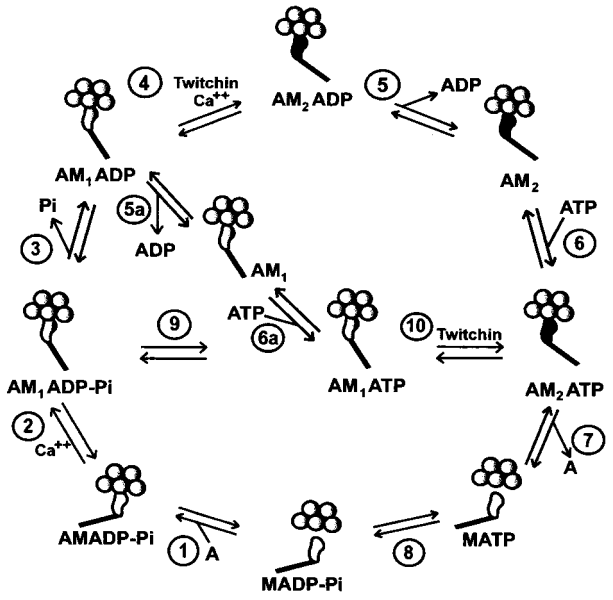


FIGURE 7 A cross-bridge scheme for catch muscle. A, actin; M, myosin. Myosin is shown in three conformations: pre-power stroke (M), intermediate force-generating (M_1), and final force-generating (M_2). These are represented as different angles of the lever arm and different shadings of the myosin head. It is postulated that the isomerization between AM_1 and AM_2 is dependent on calcium binding to myosin and the phosphorylation state of twitchin. At low [Ca^{2+}], in the absence of twitchin phosphorylation, reactions 4 and 10 are inhibited. This traps the cross-bridge in the AM_1 conformation and results in catch force maintenance. Phosphorylation of twitchin relieves this inhibition. See text for details. Note that the scheme assumes that the large conformational changes that lead to force generation do not occur simultaneously with release of the products of ATP splitting; rather, the conformational changes are stabilized by the subsequent release of products (Jontes and Milligan; 1997).

(reaction 4) is controlled both by calcium binding and phosphorylation of twitchin. When calcium is bound to AM_1ADP, the rate constant would be relatively fast and independent of the phosphorylation state of twitchin. When calcium is removed from AM_1ADP, the isomerization to AM_2ADP is dependent on whether or not twitchin is phosphorylated. When twitchin is phosphorylated, the isomerization proceeds rapidly, but when twitchin is not phosphorylated, isomerization of calcium-free AM_1ADP to AM_2ADP is essentially prevented. This could occur if the unphosphorylated twitchin interacts with the calcium-free myosin head in such a way as to block the structural rearrangement leading to movement of the lever arm of myosin to its final position. Such an interaction could be analogous to that of cardiac myosin-binding protein C (MyBP-C) and the S2 portion of myosin close to the lever arm (Gruen and Gautel, 1999). The interaction appears to modulate contractility of the muscle (Kunst et al., 2000) and is abolished by phosphorylation of MyBP-C (Gruen et al., 1999).

The model shows the following scenario for a catch contraction. An increase in intracellular [Ca^{2+}] leads to

calcium binding to myosin, cross-bridge attachment and fast cycling as shown in reactions 1–8. When [Ca^{2+}] decreases and unbinding of Ca^{2+} from myosin occurs, some cross-bridges are trapped in AM_1ADP (the catch state) since the isomerization of AM_1ADP to AM_2ADP is inhibited. Phosphorylation of twitchin allows the isomerization (reaction 4) to proceed with completion of the cycle. This results in the relaxation of force and the turnover of myosin bound ADP associated with reactions 4–8.

There are several results from the experiments described here that suggest that the scheme involving steps 1–8 in Fig. 7 needs to be expanded. This is based mainly on the finding that catch force persists after rigor treatment if twitchin is not phosphorylated. Under conditions where [Ca^{2+}] is low and twitchin is not phosphorylated, there are rigor cross-bridges that do not detach upon addition of ATP, even though ATP binds and is split to ADP and Pi. This suggests that the structural conformation of myosin that is associated with the catch state persists under rigor conditions. There is also evidence that both the intermediate and final force-generating conformations of myosin exist in rigor conditions in vertebrate smooth muscle fibers (Gollub et al., 1999). We have therefore included a rigor state designated AM_1 to the scheme shown in Fig. 7. The next question is what happens when AM_1 binds ATP. Our data suggest that if twitchin is not phosphorylated, ATP is split without detachment of the cross-bridge (reaction 9), and the muscle remains in catch. If twitchin is phosphorylated, it is likely that AM_1ATP makes the transition to AM_2ATP (reaction 10) followed by detachment, etc. Of course, ATP binding to AM_2 would result in rapid detachment of the cross-bridge, and this could account for the initial rapid relaxation of some of the force seen upon addition of ATP to the rigor muscle.

The model is also consistent with the observed effects of MgADP. The cross-bridge in the AM_1 state stays attached (in catch) whether it binds MgATP or MgADP. The force response of the catch cross-bridge is therefore independent of any competition between MgATP and MgADP binding to the rigor cross-bridge. However, addition of MgADP to AM_2 would delay the decrease in force seen upon addition of MgATP, but does not cause myosin to revert to AM_1. This would account for the slowing of the initial relaxation by MgADP as shown in Fig. 3 without an effect on the fraction of cross-bridges in catch. The muscle appears to enter into the catch state independently of the ADP concentration, and also to relax catch force even at high ADP concentrations when twitchin is phosphorylated. Such a mechanism is well suited to the effective functioning of a muscle, which must maintain high force and resistance to stretch for very long periods of time and must be able to relax when required.

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