Regulation of Catch Muscle by Twitchin Phosphorylation: Effects on Force, ATPase, and Shortening

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ABSTRACT Recent experiments on permeabilized anterior byssus retractor muscle (ABRM) of *Mytilus edulis* have shown that phosphorylation of twitchin releases catch force at pCa $>$ 8 and decreases force at suprabasal but submaximum [Ca^{2+}]. Twitchin phosphorylation decreases force with no detectable change in ATPase activity, and thus increases the energy cost of force maintenance at subsaturating [Ca^{2+}]. Similarly, twitchin phosphorylation causes no change in unloaded shortening velocity (*Vo*) at any [Ca^{2+}], but when compared at equal submaximum forces, there is a higher *Vo* when twitchin is phosphorylated. During calcium activation, the force-maintaining structure controlled by twitchin phosphorylation adjusts to a 30% *Lo* release to maintain force at the shorter length. The data suggest that during both catch and calcium-mediated submaximum contractions, twitchin phosphorylation removes a structure that maintains force with a very low ATPase, but which can slowly cycle during submaximum calcium activation. A quantitative cross-bridge model of catch is presented that is based on modifications of the Hai and Murphy (1988. *Am. J. Physiol.* 254:C99–C106) latch bridge model for regulation of mammalian smooth muscle.

INTRODUCTION

The catch state in invertebrate smooth muscle is characterized by the maintenance of force with a very low energy demand (Nauss and Davies, 1966; Baguet and Gillis, 1968) and the absence of an "active state" (Jewell, 1959), as measured by the ability to redevelop force after a quick release. In the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*, calcium activates contraction by direct binding to myosin (Kendrick-Jones et al., 1970; Szent-Györgyi and Chantler, 1994). Cholinergic nerve stimulation gives rise to an elevated intracellular calcium concentration and an initial phasic contraction associated with a relatively high energy usage. As the calcium concentration falls to near resting levels (Atsumi and Sugi, 1976; Ishii et al., 1989), there is a progression to the energetically economical catch state. If the muscle is in catch, then serotonergic nerve activation results in rapid relaxation. If serotonergic nerves are activated along with cholinergic nerves, then the contraction is only phasic, and no catch ensues.

Recently we have shown that the catch state is regulated by the cAMP-dependent phosphorylation of a high molecular weight (~600 kDa) protein (Siegman et al., 1997), and we have identified this protein as the *Mytilus* isoform of twitchin (Siegman et al., 1998). In short, our studies on both permeabilized and intact ABRM indicate that when twitchin is phosphorylated by A-kinase, rapid relaxation of catch force occurs. Furthermore, the presence of phosphorylated twitchin prevents the development of catch when calcium concentration is lowered after activation of the muscle. This tight connection between the phosphorylation state of twitchin and the presence or absence of the catch state suggests that twitchin has a central role in regulating the structure(s) responsible for catch force maintenance.

A surprising aspect of our recent work is the finding that in permeabilized ABRM the phosphorylation of twitchin causes a decrease in force output at suprabasal but submaximum calcium concentrations (Siegman et al., 1998). The calcium concentration required for half-maximum force is almost twofold higher when twitchin is phosphorylated. In addition, it was found that at equivalent submaximum forces, the unloaded shortening velocity of the muscle was higher when twitchin was phosphorylated. These results suggested that the structures responsible for catch force may exist at higher than resting calcium concentrations, and that catch structures are likely to operate together with cycling myosin cross-bridges at calcium concentrations that give less than maximum force output. The purpose of the present study is to characterize the properties of the structures responsible for the maintenance of the force that is dependent on twitchin phosphorylation.

MATERIALS AND METHODS

Solutions

Artificial seawater

Artificial seawater (ASW) consisted of the following: 10 mM KCl; 50 mM $MgCl_2$; 10 mM $CaCl_2$; 428 mM NaCl; 10 mM HEPES. The pH was 7.4.

Relaxing and activating solutions for permeabilized muscles

Relaxing solutions consisted of the following: 1 mM Mg ATP; 0.5 or 20 mM EGTA; 3 mM free Mg^{2+}; 0.5 mM leupeptin; 1 mM dithiothreitol; 5 mM P_i; 30 mM piperazine-*N,N′*-bis[2-ethanesulfonic acid] (PIPES). Ionic strength was maintained at 202 mM with 1,6-diaminohexane-*N,N,N′,N′*-

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tetraacetic acid (HDTA), and the pH was 6.8. A computer program provided by Dr. R. J. Barsotti was used to solve the multiple binding equilibria. The [Ca^{2+}] of the relaxing solutions with no added calcium was considered to be pCa $>$ 8. The activating solutions were similar to the relaxing solutions, with the exception that total EGTA was maintained at 5 mM, and the calculated free calcium concentration was varied by adjusting the amount of CaEGTA.

Other solutions

For the production of catch, relaxing solution containing 20 mM EGTA was used. Rigor solution was similar to relaxing solution, except that it contained no ATP, and the EGTA concentration was 2 mM. cAMP was used at 100 μM. The peptide inhibitor of A-kinase (Cheng et al., 1986) was obtained from Sigma Chemical Co. and was used at 10 μg/ml.

Muscle preparation

Fresh *Mytilus edulis* were obtained from Anastasi's Fish Market (Philadelphia, PA). The mussels were housed in an aquarium containing aerated and recirculating seawater (Instant Ocean) at 4°C until they were sacrificed, a period usually no longer than 3 weeks. The ABRM were isolated as previously described (Siegman et al., 1997). Muscle bundles 0.2–0.4 mm in width were isolated and mounted in stainless steel holders and incubated in aerated ASW at 20°C before use. The muscles were permeabilized by incubation in 1% Triton X-100 in rigor solution for 30 min and then rinsed in rigor solution before further experimental manipulation. All experiments were done at 20°C.

Mechanical measurements

For all measurements of force, muscle length was adjusted to slack length, *Ls*, which coincides with *Lo* (Siegman et al., 1997). Routine measurements of force production were made on a myograph described previously (Siegman et al., 1984). For the determination of unloaded shortening velocity, *Vo* (Edman, 1979), muscle bundles 2–3 mm in length and 0.2 mm wide were attached to the vane of a servomotor (Cambridge) and an Akers 801 force transducer. Quick release steps ranging in length from 3% to 12% *Lo* were generally employed, and the time required for the onset of force redevelopment following unloaded shortening was measured. *Vo* was taken as the slope of the line relating distance released versus time.

ATPase activity

To measure ATPase, permeabilized muscles were transferred from rigor solution to relaxing and activating solutions containing 1 mM ATP, 5 mM P_i, 0.2 mM P^1,P^5-di(adenosine-5′)pentaphosphate (AP_5A), 5 mM sodium azide, and 6.7 μCi/ml of [^{3}H]ATP. At 5-min intervals during rest and after activation at various calcium concentrations, aliquots of the bathing media were collected. In the absence of an ATP regenerating system, any ATPase activity would result in net ADP formation, thereby increasing the ADP concentration in the solution. Measurement of the rate of appearance of [^{3}H]ADP in the solution was accomplished by separation of ADP from ATP by HPLC, with collection and liquid scintillation counting of fractions (Vyas et al., 1994). The change in metabolite concentrations was minimized by using a large volume of incubation solution compared to muscle volume. ATPase activity was highest in pCa 5, and even under these conditions, there was less than a 1% (0.89% $\pm$ 0.07%, $N = 23$) decrease in the ATP concentration of the bathing solution. At the end of the procedure, the volume of the muscle was determined from the tritium content of the blotted muscle compared to a known volume of the incubating solution. In some muscles, twitchin was thiophosphorylated by incubation in rigor solution in the presence of 100 μM adenosine 5′-*O*-(γ-thiotriphosphate) (ATPγS) (Boehringer) and 100 μM cAMP, followed by several washes in a rigor solution before calcium activation.

Statistics

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using either the *t*-test, one way ANOVA or, when necessary, the nonparametric Mann-Whitney rank sum test.

RESULTS

Effect of cAMP on force and ATPase activity at varying calcium concentrations

The effect of cAMP on the suprabasal ATPase rate was determined during isometric force maintenance at various calcium concentrations. As shown in Fig. 1, this was done by directly comparing the ATPase during an initial 5 min of force maintenance (control) to that during the 5 min following the addition of cAMP in the same muscle. Previous studies have shown that such an addition of cAMP under these conditions leads to phosphorylation of twitchin (Siegman et al., 1997, 1998). Fig. 1 shows that when the muscle is maximally activated in pCa 5, phosphorylation of twitchin caused no significant change in either force output or ATPase activity.

A similar experimental paradigm was followed for muscles submaximally activated in pCa 6 (Fig. 2). At this calcium concentration, force, which was initially ~40% of

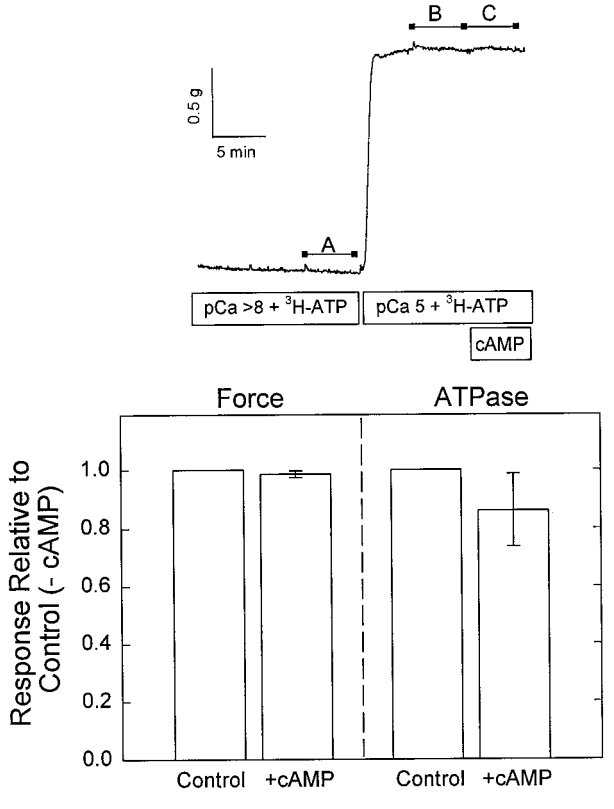

FIGURE 1 Effect of cAMP on force and suprabasal ATPase at pCa 5. The top panel shows a typical force trace and the intervals (5 min) over which the increase in [ADP] was used to determine ATPase activity. B–A was the control suprabasal ATPase, and C–A was suprabasal ATPase after the addition of cAMP (100 μM). The lower panel shows force and ATPase normalized to the control in each muscle. Data are means $\pm$ SEM, $N = 6$.

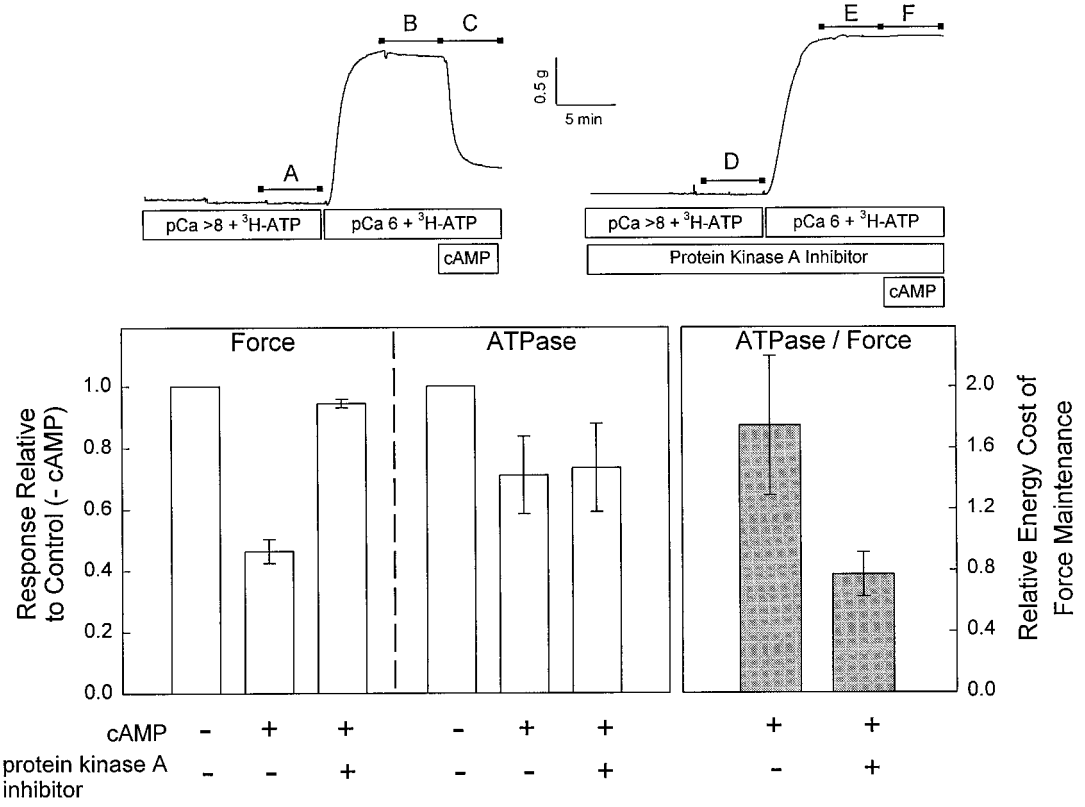

FIGURE 2 Effect of cAMP on force, suprabasal ATPase, and ATPase/force at pCa 6 in the presence and absence of a peptide inhibitor of A-kinase. The top panels show typical force responses and the intervals (5 min) over which ATPase was measured. B–A and E–D are the control suprabasal ATPases in the absence and presence of the peptide inhibitor. C–A and F–D are the suprabasal ATPases after the addition of cAMP in the absence and presence of the peptide inhibitor. Force and ATPase are shown normalized to the control ATPase before the addition of cAMP in each muscle. Also shown is the energy cost of force (ATPase/force) in the presence and absence of the A-kinase inhibitor. Data are means ± SEM, and $N = 7$ and 9, respectively, for muscles with and without the A-kinase inhibitor.

maximum, was significantly reduced upon addition of cAMP. There was also an apparent decrease in ATPase activity that approached but did not achieve statistical significance ($p = 0.06$). We recognized the possibility that the ATPase activity might decrease over the time course of the activation, and that this might complicate determination of the true effect, if any, of phosphorylation of twitchin on ATPase activity. For this reason, we performed an experiment in which cAMP was added in the presence of the synthetic peptide inhibitor of protein kinase A. We have previously shown that this procedure very effectively blocks both the cAMP-mediated phosphorylation of twitchin and the associated decrease in force at submaximum calcium concentrations (Siegman et al., 1998). Fig. 2 shows that, indeed, in the presence of the A kinase inhibitor, force production at pCa 6 was changed only minimally by the addition of cAMP. Under these conditions, the suprabasal ATPase activity was almost identical in magnitude to that observed in the absence of the A-kinase inhibitor. In these experiments, which are identical except for the presence of the A-kinase inhibitor, the large difference in force output is not matched by a large difference in ATPase activity. Taken together, these results suggest that at this intermediate calcium concentration, phosphorylation of twitchin leads to a diminution of force production, with little or no effect on ATPase activity. Fig. 2 also shows that the energy cost of force maintenance (relative ATPase activity/relative force) is significantly higher when twitchin is phosphorylated and force is reduced.

We have previously shown that in the presence of cAMP, twitchin can be specifically thiophosphorylated with ATPγS (Siegman et al., 1997) and that such a thiophosphorylation prevents the appearance of the catch state when calcium is removed from an activated muscle. Fig. 3 shows that when twitchin is thiophosphorylated before activation in pCa 6, the addition of cAMP does not lead to a reduction in force. At intermediate calcium concentrations, irreversible thiophosphorylation of twitchin appears to set the force output to the lower value, which is normally achieved by cAMP-dependent phosphorylation of twitchin. Fig. 4 shows a quantitative summary of the effect of prethiophosphorylation of twitchin on force output at pCa 6.0–6.1 relative to pCa 5. cAMP-dependent prethiophosphorylation of twitchin does result in a significantly lower relative force output at this intermediate calcium concentration. ATPase activity was also measured in the same set of muscles at identical times after activation, and the data are shown as absolute suprabasal ATPase values in Fig. 4. Even though prethio-

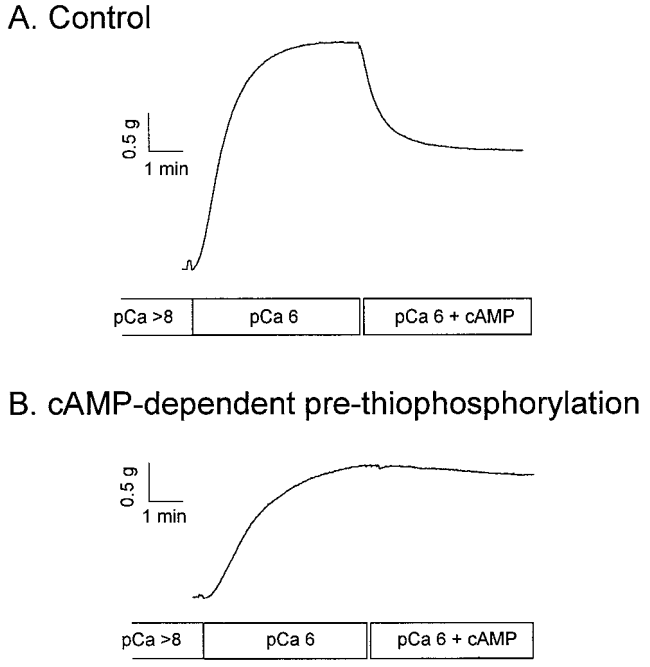

FIGURE 3 The absence of an effect of cAMP on submaximum force when twitchin is thiophosphorylated before calcium activation. (*A*) The typical decrease in force seen upon addition of cAMP (100 μM) in pCa 6. (*B*) The response from a muscle in which twitchin was thiophosphorylated before activation by incubation in rigor solution in the presence of 100 μM ATPγS and 100 μM cAMP followed by several washes in a rigor solution. The addition of cAMP has no effect on force.

phosphorylation of twitchin reduced relative force by ~50%, there is no significant effect on ATPase activity. Also shown are the values for energy cost of force maintenance. By a nonparametric test, there is a significantly higher energy cost of force maintenance at this intermediate calcium concentration when twitchin is prethiophosphorylated.

Fig. 5 shows force production and ATPase activity measured during cumulative increases in calcium concentration in individual muscles. From the calculated A_{50} values (Tallarida and Murray, 1987) for each set of data, the calcium concentration that produced half-maximum force increased significantly from pCa 6.13 $\pm$ 0.02 to pCa 5.99 $\pm$ 0.05 as a result of prethiophosphorylation of twitchin, whereas there was no significant difference in the dependence of ATPase on calcium concentration in the two conditions. Fig. 6 shows ATPase as a function of force. At intermediate forces (e.g., 0.2–0.6 max), there is a larger ATPase for a given force when twitchin is thiophosphorylated, but at higher forces (>0.6 max) the curves merge. In other words, twitchin phosphorylation alters the economy of force maintenance at calcium concentrations that give submaximum force, but not at force-saturating calcium concentrations.

On the whole, the data on ATPase activity and force suggest that cAMP-mediated phosphorylation of twitchin results in the loss of a force-maintaining structure with little or no change in ATPase activity at intermediate calcium concentrations. This suggests that in the absence of cAMP when twitchin is dephosphorylated, an "extra" force is maintained by a structure whose ATPase activity is quite low compared to "normal" cross-bridge cycling.

Effect of cAMP on force and ATPase during a catch protocol

We have previously described a protocol in the permeabilized ABRM that mimics the catch state seen in intact muscles (Siegman et al., 1997). In the protocol illustrated in Fig. 7, the muscle is activated in pCa 5 (5 mM total EGTA) and then transferred to a solution containing pCa > 8 (20 mM total EGTA, no added calcium). In the low calcium solution, the force shows an initial rapid decline followed by a much slower rate of relaxation. During this very slow phase of relaxation, force is maintained for many minutes (>10), and little, if any, force redevelopment results after a quick release (see Siegman et al., 1997). This is catch. Importantly, most of this slowly relaxing force can be rapidly dissipated by the addition of cAMP and the resulting A-kinase-mediated phosphorylation of twitchin. It was of interest to compare the energy requirements of catch force maintenance to that of the "extra" force maintained at subsaturating calcium concentrations when twitchin is dephosphorylated. Fig. 7 shows the ATPase of muscles during maximum activation in pCa 5, during catch, and after release of catch with cAMP. There is a very large decrease in ATPase activity when the muscle is put into catch, and no further significant change upon subsequent treatment with cAMP. Furthermore, there is a much larger decrease in relative ATPase than in the tension-time integral in the transition from activation in pCa 5 to catch. In paired measurements, the energy cost of force maintenance in catch is only 26 $\pm$ 6% of that during activation in pCa 5. It thus seems that the catch state shares two important characteristics with the "extra" force maintained at submaximal calcium concentrations: 1) a high economy of force maintenance and 2) disappearance when twitchin is phosphorylated.

Unloaded shortening velocity

The absence of an effect of phosphorylation of twitchin on ATPase activity at high and intermediate calcium concentrations raised questions about whether the shortening properties of the muscle might be affected. The muscles were activated with the appropriate calcium concentration, and when steady-state force was achieved, *Vo* was determined. The mean *Vo* (*Lo*/s) at pCa 5 is 0.035 $\pm$ 0.003 (N = 17); Fig. 8 shows how both force and *Vo* varied as a function of calcium concentration. There was a significant dependence of both of these parameters on calcium concentration, and, interestingly, at the calcium concentrations tested, relative force and relative *Vo* appeared to vary proportionally.

To determine the effect of phosphorylation of twitchin on *Vo*, measurements were made at a given calcium concentration on the same muscle before and after the addition of

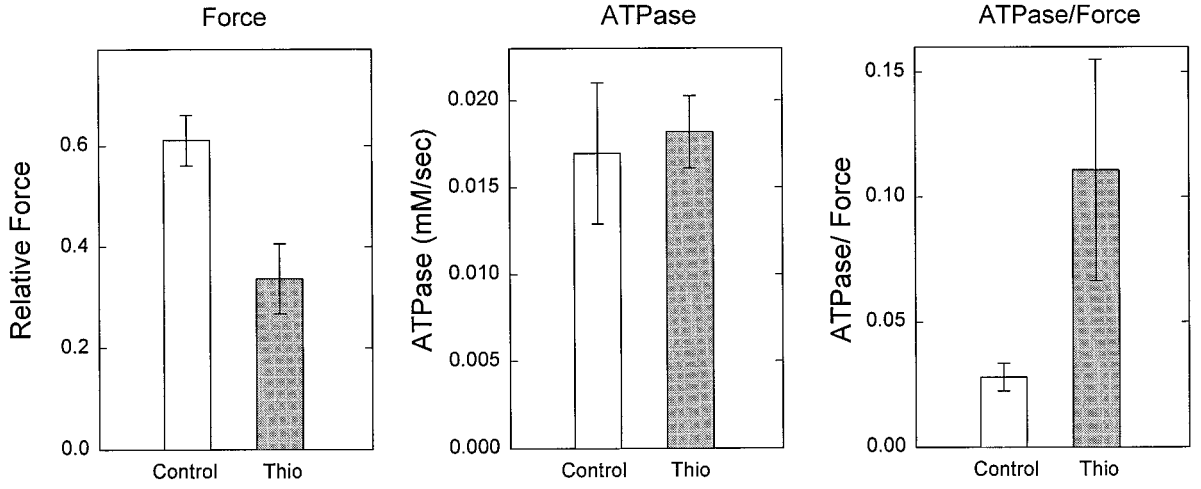

FIGURE 4 Effect of thiophosphorylation of twitchin on force, ATPase, and energy cost of force (ATPase/force) at pCa 6.1–6.0. Force is reported relative to maximum force in pCa 5. ATPase was measured during 5-min intervals and is shown as the difference between ATPase at rest and ATPase during activation. ATPase is reported as mmol ATP split/liter muscle volume/s. In one group of muscles, twitchin was thiophosphorylated (Thio) as described in Materials and Methods. Control muscles were not so treated. The energy cost of force output is the ATPase/relative force. Data are means ± SEM. Each group contains 10 muscles, with six of the 10 in each group activated at pCa 6.1, and the others were activated in pCa 6.0.

cAMP. The results are shown in Fig. 9. For muscles activated at pCa 6.3, the addition of cAMP resulted in a significant decrease in force but had no significant effect on the corresponding *Vo*. When the calcium concentration was increased (pCa 5.88) so that force equaled that before the addition of cAMP, there was a significantly faster *Vo*. These results show that at an equivalent submaximum force, the unloaded shortening velocity of the muscle was higher when twitchin was phosphorylated, and they are similar to those reported previously for muscles activated at pCa 6 (Siegman et al., 1998). We have also found that there is no change in *Vo* at pCa 6 when force is decreased by the addition of cAMP (data not shown). In muscles activated in pCa 5, the addition of cAMP caused only a small decrease in force and no significant change in *Vo* (Fig. 9, *lower panels*).

Do cAMP-dependent force-bearing structures adjust during muscle shortening and thereby maintain force at the shorter muscle length?

The fact that the structures responsible for maintenance of the cAMP-dependent force at intermediate calcium concentrations could be removed with little or no effect on ATPase activity raised the question of whether these structures (be they myosin cross-bridges or perhaps some other interfilament linkages) have the ability to cycle and adjust during shortening. Specifically, the experiments were designed to determine whether the structures have the ability to adjust during muscle shortening so that they may maintain force at a new shorter muscle length. Muscles were activated at intermediate calcium concentrations and then subjected to quick releases in length ranging from 10% to 30% *Lo*. Force was allowed to redevelop, and then cAMP was added to the bathing medium. The rationale for this experiment is as follows: the large releases would be expected to cause the buckling and/or detachment of all force-maintaining linkages between filaments. The cross-bridges would cycle and give rise to force redevelopment at the new length. Any subsequent reduction in force upon the addition of cAMP would indicate that some of the redeveloped force maintenance was due to the structures that are dependent on the state of twitchin phosphorylation. This would mean that the linkages responsible for the cAMP-dependent force would be capable of resetting to maintain force at the new shorter length, and then could be removed by the addition of cAMP. Fig. 10 shows typical results for muscles activated at pCa 6.3. Similar results were obtained for muscles activated at pCa 6 (data not shown). Almost complete recovery of force occurred after 10% and 15% *Lo* releases in length (Fig. 10, *C* and *D*, respectively). After a 30% *Lo* release (Fig. 10 *E*), the mean force recovery was 58 ± 5% ($n = 6$). After the 30% *Lo* release and before force recovery, force remained at zero for ~60 s while the muscle freely shortened. In every case, after redevelopment of force after shortening, the addition of cAMP resulted in a large decline of force that was similar to the relative decrease in force seen in control muscles not subjected to shortening (compare Fig. 10, *C–E*, with Fig. 10 *B*). Importantly, cAMP applied to a muscle at a time somewhat before the quick release also resulted in a marked decline in force (Fig. 10 *A*). These results suggest that the cAMP-dependent force-maintaining structures are present before the shortening, can detach upon shortening, and can subsequently reattach (cycle) to maintain newly developed force at the shorter muscle length.

DISCUSSION

Effects of twitchin phosphorylation on ATPase and V_o

Simply stated, we have found no significant effects of twitchin phosphorylation on either *Vo* or ATPase of the

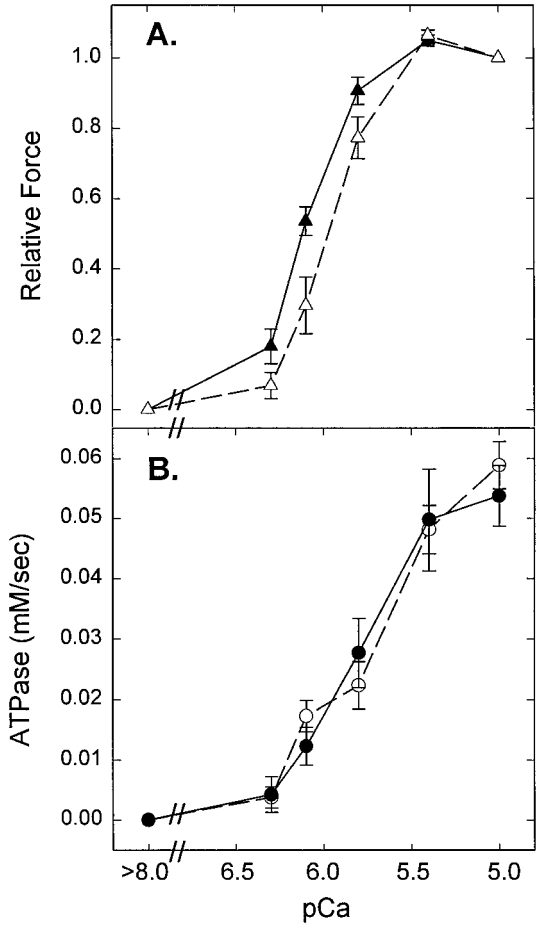

FIGURE 5 Force and ATPase as a function of calcium concentration in the presence or absence of twitchin thiophosphorylation. Calcium was increased cumulatively from no added calcium (pCa > 8) to pCa 5 in each muscle. Force in *A* is reported relative to that at pCa 5. ATPase in *B* is reported as mmol ATP split/liter muscle volume/s. The basal ATPase at pCa > 8 was subtracted from all values. In one group of muscles, twitchin was thiophosphorylated (○, △) as described in Materials and Methods, whereas control muscles were not so treated (●, ▲). Data are means ± SEM, $N = 6$.

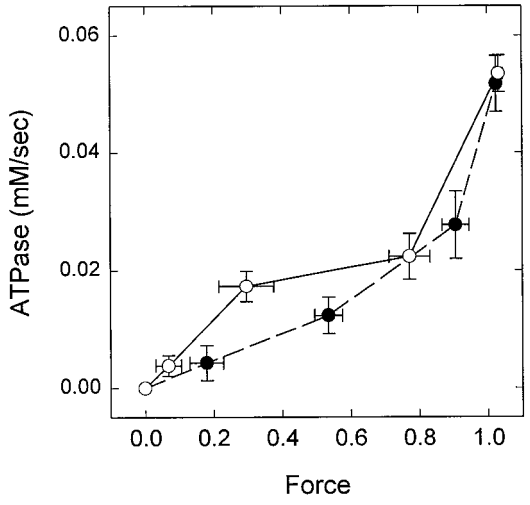

FIGURE 6 Effect of thiophosphorylation of twitchin on the relationship between ATPase and force. The data are from the experiment described in Fig. 5, with the results from pCa 5.3 and 5.0 combined. ○, Twitchin thiophosphorylated; ●, controls. Data are means ± SEM, with $N = 6$, except for maximum values, where $N = 12$.

ABRM at any of the calcium concentrations tested. However, the absence of changes in these parameters in the face of the relatively large decrease in force caused by this phosphorylation at intermediate calcium concentrations provides important insight into the characteristics of the structure responsible for the variable force output.

The results from ATPase measurements suggest that when twitchin is dephosphorylated, some force is maintained by a structural element that has a very low ATPase activity compared to normal cross-bridge cycling. This suggests that the structure may be capable of only slow cycling, and that it would not be able to adjust rapidly when the muscle was allowed to shorten. On these grounds, one would predict that treatment with cAMP and phosphorylation of twitchin might lead to an increase in *Vo*, because of the removal of an internal resistance to shortening provided by the slow cycling force-maintaining structures. The data show, however, no significant change in *Vo* with phosphorylation of twitchin. It must be noted, however, that treatment with cAMP at submaximum calcium concentrations results in a decrease in force output, so *Vo* is measured in the presence of fewer force generators. If a decrease in resistance to shortening were balanced by the effect of the loss of force generators, then no change in *Vo* would occur. Indeed, an increase in calcium concentration in the presence of cAMP that is sufficient to match the original force in the absence of cAMP does result in a higher *Vo*. Thus, at the same relative submaximum force, there is a faster *Vo* when twitchin is phosphorylated than when it is dephosphorylated. It is not known whether the higher *Vo* at the same relative force after twitchin phosphorylation results from an alteration in internal loading as described above, or whether it is due to the inherent dependence of *Vo* on calcium concentration (see Fig. 8), as has also been shown in other muscles (for a review, see Moss, 1992).

The change in the relationship between ATPase and force output caused by twitchin phosphorylation is strikingly similar to the change in the relationship between *Vo* and force. At intermediate calcium concentrations where force output changes with phosphorylation, equivalent force output is associated with a significantly higher ATPase when twitchin is phosphorylated.

The structure responsible for catch force adjusts after shortening

We have shown here that the structure that is responsible for the maintenance of this extra and economical force during submaximum contractions has the ability to adjust during muscle shortening, and subsequently to maintain force at the shorter muscle length. This property may reflect an

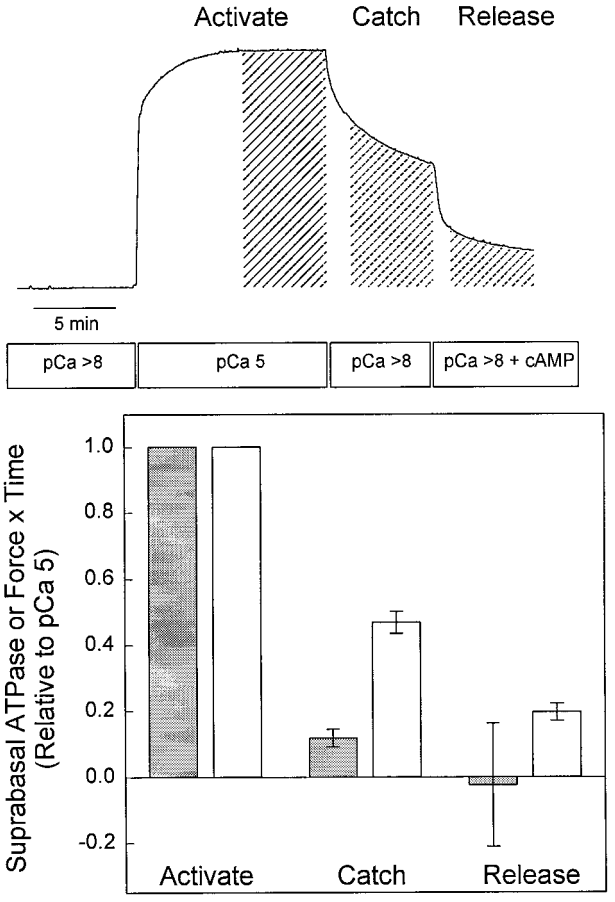

FIGURE 7 Force and ATPase during activation, catch, and release of catch by cAMP. The upper panel shows the protocol and a typical force trace. Muscles were activated in pCa 5 (Activate) and transferred to pCa > 8 (Catch), and then cAMP was added (Release). The hatched areas show the intervals during which ATPase and the force-time integral (force × time) were measured. The lower panel shows the data for suprabasal ATPase (*filled bars*) and force-time integral (*open bars*). Both parameters are normalized to those in pCa 5 for each muscle. Data are means ± SEM, $N = 5$.

ability to cycle, such that it maintains force under isometric conditions, readjusts (detaches) after a shortening, and maintains the redeveloped force (reattaches) at the new shorter length. It is important to note that the readjustment of the structure does not appear to occur unless the calcium concentration is suprabasal, and there is a calcium-driven shortening and force redevelopment. For example, Jewell (1959) has shown that there is minimal force redevelopment after a quick release when the intact ABRM is in catch, and that subsequent stretch to the original length restores the original force. These early results were confirmed in the system used for these experiments by Siegman et al. (1997). At the very low (near-basal) calcium concentration associated with catch (Ishii et al., 1989), the force-maintaining structure does not adjust upon shortening, but in the presence of calcium-driven cross-bridge cycling, the structure responsible for economical force maintenance does, indeed, readjust (cycle?) to maintain force at the new length.

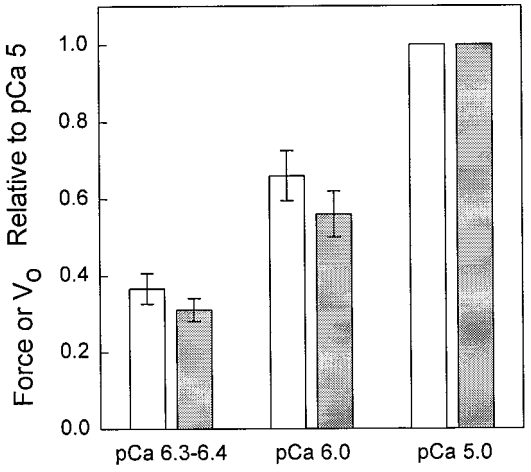

FIGURE 8 Dependence of unloaded shortening velocity (*Vo*) and force on calcium concentration. Muscles were activated at the calcium concentration shown, and *Vo* was determined at steady-state force. The same measurements were then made in pCa 5. Both force (*filled bars*) and *Vo* (*open bars*) were normalized to the values at pCa 5. Data are means ± SEM, $N = 8–17$.

Hypotheses concerning the mechanism of catch

The basis of force maintenance in catch is not known, but two major hypotheses concerning the mechanism made over 30 years ago are still considered today. Essentially the main difference between these two hypotheses is whether catch force is maintained by the structures that develop the force

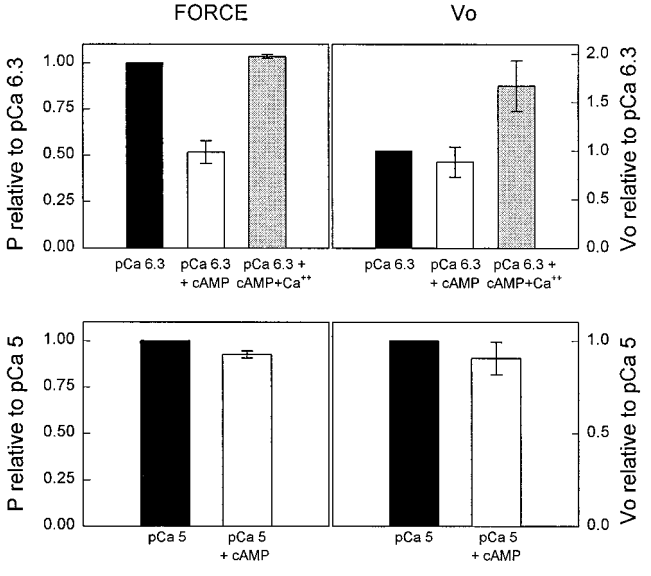

FIGURE 9 Effect of cAMP on unloaded shortening velocity (*Vo*) and force output at different calcium concentrations. (*Upper panels*) Muscles activated at pCa 6.3 (*black bars*) followed by the addition of cAMP (*white bars*). The calcium concentration in the bathing medium containing cAMP was then increased until the force equaled that before the addition of cAMP (*gray bars*). All values are relative to those at pCa 6.3 without cAMP. (*Lower panels*) Muscles were activated at pCa 5, and force and *Vo* were measured before (*black bars*) and after (*white bars*) the addition of cAMP. $N = 12–14$.

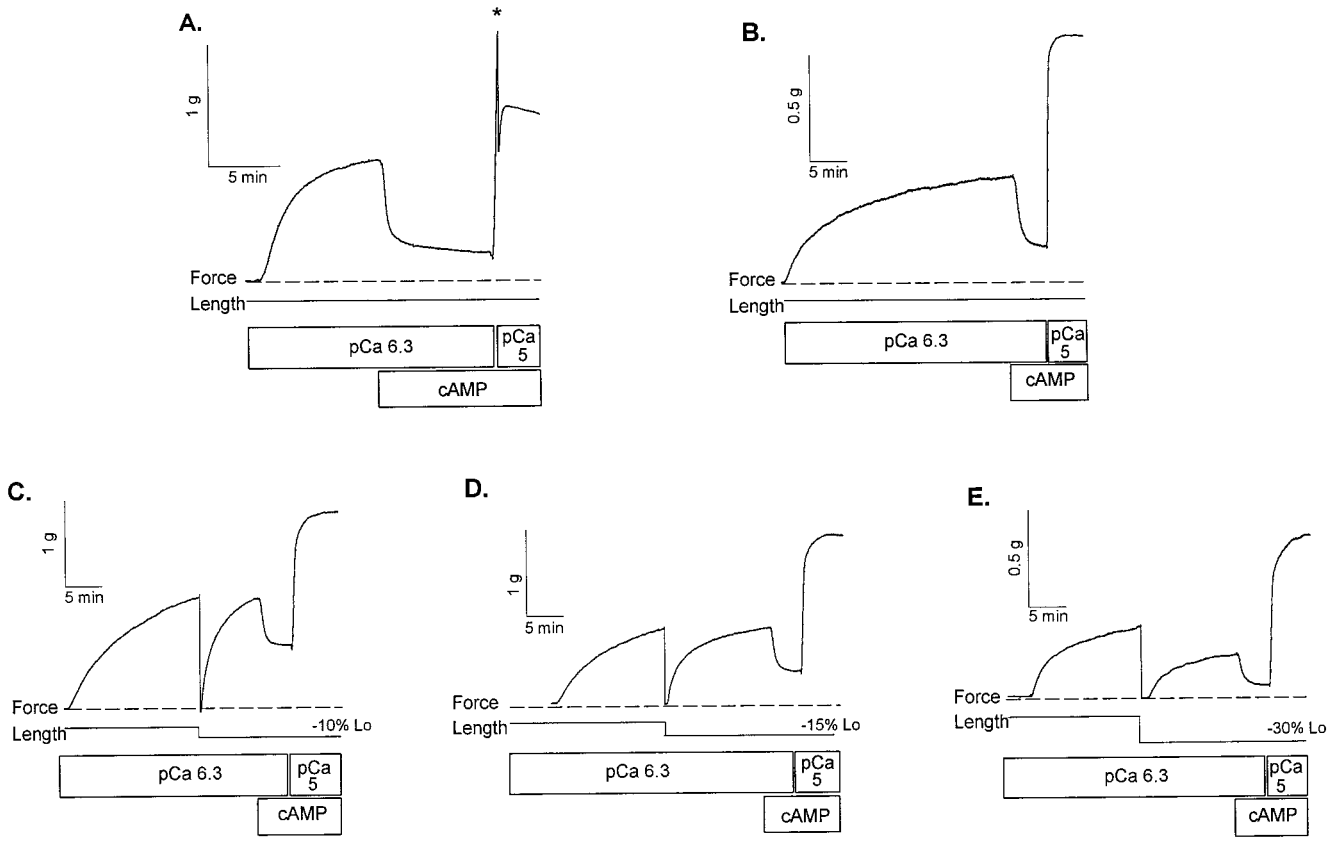

FIGURE 10 Effect of muscle shortening and force redevelopment on cAMP-dependent force output at pCa 6.3. Control experiments (no change in muscle length) were performed to show that cAMP added at both early (8 min) (*A*) and late (30 min) (*B*) times after activation in pCa 6.3 causes a decrease in force output. At *, there was a twofold decrease in sensitivity of force measurement. (*C*, *D*, and *E*) The muscles were subjected to quick releases of 10%, 15%, and 30% *Lo*, respectively, ~15 min after activation of the muscle. After redevelopment of force, cAMP was added to the bathing medium, and in every case, force decreased.

(myosin cross-bridges) and which have a very slow rate of detachment during catch (Lowy et al., 1964), or rather by other structures that link thick and thin filaments after force production by myosin cross-bridges (Ruegg, 1961).

Twitchin, a mini-titin, plays a central role in the control of catch, and the similarities in structure between twitchin and titin suggest that they may share some functional properties. In this regard it is interesting that a titin fragment consisting of linked class 1 and class 2 motifs inhibits actin filament sliding in an in vitro motility assay, possibly by tethering actin to the nitrocellulose-coated coverslip (Li et al., 1995). Because twitchin is associated with the thick filament in catch muscle (Vibert et al., 1993) and contains linked class 1 and class 2 motifs thought to mediate some aspects of titin binding to actin, it might provide a mechanical link between the thick and thin filaments independent of the myosin cross-bridge. In addition, it has been shown that the titin interaction, with respect to slowing in vitro motility of regulated thin filaments, is calcium dependent (Kellermayer and Granzier, 1996). So it is conceivable that twitchin itself may be the mechanical link responsible for catch force maintenance. Phosphorylation of twitchin might cause detachment of the link, but the details of how different amounts of catch force might be present at different calcium concentrations and how the catch force-maintaining structure might be able to cycle to different extents at different degrees of calcium activation are certainly not known.

Of course, it is also possible that the catch force-maintaining structure is the myosin cross-bridge. If this is the case, then phosphorylation of twitchin would have to affect the kinetic parameters of the cross-bridge. Twitchin is distributed along the whole length of the thick filament in catch muscle (Vibert et al., 1993), and there is approximately one twitchin for every 15 myosin molecules (Siegman et al., 1997). Because twitchin is a long (0.2–0.25 μm) rodlike molecule, it is possible that it lies on the surface of the thick filament and that one molecule interacts directly with many different myosin molecules. Such an interaction would provide a means for the phosphorylation of a single twitchin molecule to control the kinetic parameters of many myosin molecules.

Because there is a rapid decrease in force during catch and a decrease in economy of force maintenance at submaximum calcium concentrations, it seems that phosphorylation of twitchin might cause an increase in the rate constant for cross-bridge detachment. But there does not appear to be such an increase in detachment rate constant for cross-bridges at calcium concentrations that give rise to

maximum force output, because neither force output nor ATPase activity changes with twitchin phosphorylation. The difference in these two situations may depend on whether the cross-bridge in question has calcium bound to it. The calcium-free attached cross-bridge present during catch may have a slow detachment rate constant when twitchin is dephosphorylated, and a much faster detachment rate constant when twitchin is phosphorylated. The identification of the calcium-free, attached cross-bridge as the structure affected by twitchin phosphorylation would explain why there is no effect of twitchin phosphorylation on either ATPase or force at saturating calcium concentrations. Under such conditions there would be no calcium-free attached cross-bridges. At intermediate calcium concentrations, some myosin has calcium bound, but there may also be calcium-free myosin attached to actin.

A quantitative model of catch

The similarity of some aspects of the mechanical behavior of mammalian smooth muscle to the catch state in molluscan smooth muscle was noted more than 30 years ago (Somlyo and Somlyo, 1967), and the designation of a noncycling, force-maintaining cross-bridge as a latch bridge in mammalian smooth muscle was obviously based on similarities to catch force-maintaining structures in invertebrate muscle (Dillon et al., 1981). Hai and Murphy (1988) presented a quantitative model for cross-bridge regulation in mammalian smooth muscle, and although the model does not fully account for all of the details of regulation (e.g., see Gerthoffer, 1987; McDaniel et al., 1992), it is consistent with much of the experimental data. The Hai-Murphy latch bridge model also provides a framework upon which a model for catch can be based. In the application of the Hai-Murphy model to catch muscle, two modifications are necessary: 1) replacement of the regulation by phosphorylation and dephosphorylation of the myosin light chain (mammalian smooth muscle) with calcium binding to and release from myosin (invertebrate smooth muscle); and 2) variation in the equivalent of the detachment rate constant of mammalian myosin with dephosphorylated light chain. Yu and colleagues (Yu et al., 1997), in applying such changes to the Hai-Murphy model, found the model to be a good predictor of the mechanical responses reported by others in the intact ABRM during catch. Using the data on permeabilized ABRM described here, we also test the applicability of the Hai-Murphy model modified for catch.

The quantitative model is shown in Fig. 11. The important aspects of the model are that calcium can unbind (k_2) from an attached cross-bridge and that the calcium-free, attached cross-bridge (AM), which still maintains force, has a very slow detachment rate constant (k_7) compared to that of the calcium-bound myosin (k_4). Furthermore, importantly, the catch properties of the muscle are regulated in that k_7 is much slower when twitchin is dephosphorylated than when it is phosphorylated. The specific values for the various rate constants are shown in Fig. 11. The ratio between k_3 and k_4 was chosen such that ~80% of myosin would be attached at calcium saturation, and the absolute value of k_4 was chosen to give a maximum isometric ATPase of ~0.6 s^{-1} per myosin head. This is based on the data in Fig. 5, showing an ATPase of ~60 μM/s, and other studies that indicated that myosin S1 content is ~100 μM (data not shown). The value for k_7 − cAMP was chosen to approximate the time course of the fall of force in catch, and k_7 + cAMP was taken to be an order of magnitude larger, to result in the rapid release of catch when twitchin is phosphorylated. k_2 was chosen to be 0.05 s^{-1}, so that given the other rate constants, the predicted effect of twitchin phosphorylation on force and ATPase at submaximum calcium concentrations was similar to the observed behavior of the muscle. Fig. 11, *B–F*, shows predictions of the model when changes in the rate constant k_1 are made to simulate changes in calcium concentration. In Fig. 11 *B*, k_1 is increased from zero (rest) to a value that almost saturates myosin with calcium, and is then returned to zero. Force is developed and maintained when k_1 is high, and there is a slow decay of the developed force when k_1 is set back to zero. Although not shown in the figure, ATPase is high when k_1 is high and very low when force is slowly decaying. The rate of decay of force is increased dramatically if k_7 is increased to the +cAMP value. The similarities of the output of the model to the mechanical events observed in permeabilized ABRM in catch are evident when the force response in Fig. 11 *B* is compared to that shown in Fig. 7.

Fig. 11, *C–F*, shows force and ATPase simulations of the effects of adding cAMP when different amounts of myosin are saturated with calcium. A shift to the high k_7 (equivalent to phosphorylation of twitchin) only changes force at subsaturating calcium concentrations, whereas it has little or no effect on ATPase under any condition. A comparison of the simulations to the data shown in Figs. 1 and 2 demonstrates the success of the model in predicting the major aspects of the force and ATPase responses of the ABRM under maximally activated conditions, as well as at subsaturating calcium concentrations, where there is a large effect of twitchin phosphorylation on force but not on ATPase.

It is interesting that in this model, the catch cross-bridge (AM in Fig. 11 *A*) can cycle both by detachment through k_7 and by binding and unbinding of calcium through k_1 and k_2. Of note is the observation that in the absence of calcium, the loss of AM would be slow (only through k_7), and, except for an initial net AM formation upon removal of calcium, no new AM would form under calcium-free conditions. This slow loss of AM and lack of formation of new AM could account for the absence of readjustment of the catch-maintaining structure when the muscle is released at very low calcium concentrations. In contrast, at higher but subsaturating calcium concentrations, there would be a continual formation and loss of AM through k_2 and k_1, respectively, and this could lead to the readjustment of the catch-maintaining structure (AM) upon shortening, so that it maintains force at a new, much shorter muscle length. A more detailed

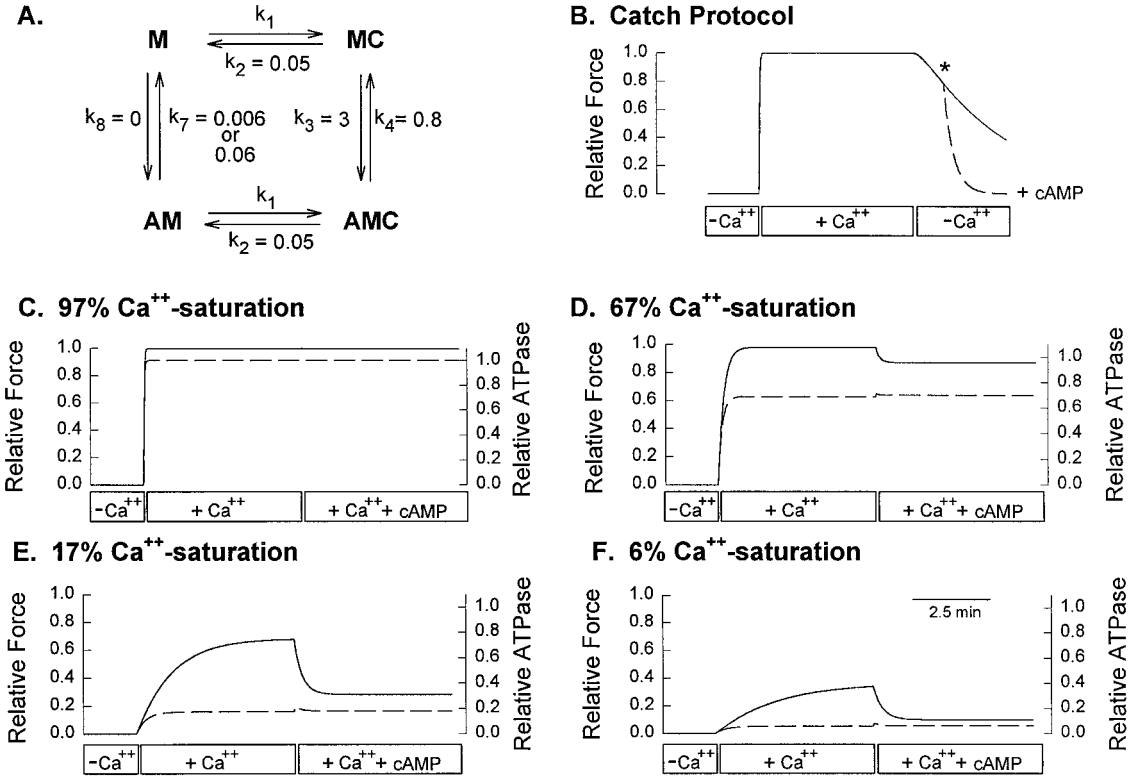

FIGURE 11 A quantitative model of catch. (*A*) A four-state model for catch based on the Hai-Murphy (Hai and Murphy, 1988) model for regulation of mammalian smooth muscle. MC and M represent myosin with and without calcium bound, respectively. Both of these states are non-force-producing (detached from actin). AMC and AM are force-producing states (attached to actin) with and without calcium bound, respectively. All rate constants are first order and are reported as s^{-1}. k_7 is 0.006 s^{-1} when twitchin is not phosphorylated and is 0.06 s^{-1} when it is phosphorylated. An increase in k_1 simulates an increase in calcium concentration. (*B*) Simulation of the force response during the catch protocol (——). k_1 was increased from 0 (− Ca^{2+}) to 1.5 s^{-1} (+ Ca^{2+}), resulting in 97% of the myosin in calcium-bound forms, and then decreased again to zero. A simulation of the effect of adding cAMP was made by increasing k_7 from 0.006 to 0.06 s^{-1} at * (– – –). (*C–F*) Simulations of the effects of the addition of cAMP (increase in k_7 from 0.006 to 0.06 s^{-1}) on force and ATPase at different levels of activation of the muscle. The level of activation was changed by varying k_1 from 1.5 s^{-1} (*C*) to 0.1 s^{-1} (*D*), 0.01 s^{-1} (*E*), and 0.003 s^{-1} (*F*). Force (——) and ATPase (– – –) are shown relative to the maximum values in *C*. Force is the sum of AMC and AM, whereas ATPase is calculated as k_4AMC + k_7AM. The time calibration applies to *B–F*.

analysis of the effect of twitchin phosphorylation on shortening characteristics would require information on the effects of strain on the kinetics of the cross-bridge cycle and on the mechanical interaction of AM and AMC during shortening.

Limitations of the quantitative model of catch

Although the quantitative model satisfactorily predicts the major mechanical and ATPase data presented here, there are certain limitations of the predictions of the model. The fall of force during catch in the permeabilized muscle is biphasic (see Fig. 7), whereas the fall in force predicted by the model during catch is essentially exponential (k_7 − cAMP), with a rate constant of 0.006 s^{-1}. To account for the very slow phase of the decay of catch force, this rate constant would have to be considerably slower. The measured ATPase, albeit low, is ~12% rather than the predicted <1% of the ATPase in pCa 5. Baguet and Gillis (1968) have also shown a low but significant suprabasal oxygen consumption during catch in the intact muscle. The higher than expected ATPase could result from a finite cycling of cross-bridges (i.e., both attachment and detachment) during catch. The finite rate of cross-bridge attachment in the absence of calcium may result from cooperative mechanisms (Weber and Murray, 1973; Somlyo et al., 1988; Vyas et al., 1992). Such a mechanism may also contribute to the slower than predicted decay of catch force. It is also important to note that the mechanism(s) that might result in the changes in detachment rate constant with phosphorylation of twitchin are not known, but could include differences in off rate of ADP from the cross-bridge (Fuglsang et al., 1993; Khromov et al., 1995) and/or ATP binding kinetics (Khromov et al., 1996), as has been described for phasic and tonic mammalian smooth muscle.

SUMMARY

The results presented here show that phosphorylation of twitchin can be considered to have the same effect during catch and during calcium-mediated submaximum contractions, namely the disabling of a structure that maintains

force with a very low ATPase requirement. During calcium-mediated contractions, the structure seems to be able to adjust during muscle shortening so that it can subsequently maintain force at a new shorter muscle length. A quantitative model of catch is presented, based on the ideas proposed by Hai and Murphy (1988) for the regulation of mammalian smooth muscle. In the model, phosphorylation of twitchin increases the rate of detachment of a calcium-free attached cross-bridge, which is equivalent to the latch bridge in the mammalian smooth muscle.

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