Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 5383–5388, April 1998
Physiology

Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle

MARION J. SIEGMAN*†, DAISUKE FUNABARA‡, SHIGEHARU KINOSHITA‡, SHUGO WATABE‡, DAVID J. HARTSHORNE§, AND THOMAS M. BUTLER*

*Department of Physiology, Jefferson Medical College, Philadelphia, PA 19107; ‡Laboratory of Aquatic Molecular Biology, University of Tokyo, Tokyo 113, Japan; and §Muscle Biology Group, University of Arizona, Tucson, AZ 85721

Communicated by Carolyn Cohen, Brandeis University, Waltham, MA, February 13, 1998 (received for review November 15, 1997)

ABSTRACT **"Catch" is a condition of prolonged, high-force maintenance at resting intracellular Ca^{2+} concentration ($[Ca^{2+}]$) and very low energy usage, occurring in invertebrate smooth muscles, including the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*. Relaxation from catch is rapid on serotonergic nerve stimulation in intact muscles and application of cAMP in permeabilized muscles. This release of catch occurs by protein kinase A-mediated phosphorylation of a high (≈600 kDa) molecular mass protein, the regulator of catch. Here, we identify the catch-regulating protein as a homologue of the mini-titin, twitchin, based on (*i*) a partial cDNA of the purified isolated protein showing 77% amino acid sequence identity to the kinase domain of *Aplysia californica* twitchin; (*ii*) a polyclonal antibody to a synthetic peptide in this sequence reacting with the phosphorylated catch-regulating protein band from permeabilized ABRM; and (*iii*) the similarity of the amino acid composition and molecular weight of the protein to twitchin. In permeabilized ABRM, at all but maximum $[Ca^{2+}]$, phosphorylation of twitchin results in a decreased calcium sensitivity of force production (half-maximum at 2.5 vs. 1.3 μM calcium). At a given submaximal force, with equal numbers of force generators, twitchin phosphorylation increased unloaded shortening velocity ≈2-fold. These data suggest that aspects of the catch state exist not only at resting $[Ca^{2+}]$, but also at higher submaximal $[Ca^{2+}]$. The mechanism that gives rise to force maintenance in catch probably operates together, to some extent, with that of cycling myosin crossbridges.**

Isometric force production in the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*, initiated by cholinergic nerve stimulation, is maintained for a prolonged period of time after cessation of stimulation. This condition, during which relaxation of force occurs at an extremely slow rate, lasting minutes, or even hours, has been termed "catch." When catch occurs, the intracellular Ca^{2+} concentration ($[Ca^{2+}]$), which was transiently elevated as a result of the stimulus, has declined to near-resting levels (1, 2). In this invertebrate smooth muscle, calcium activates contraction by direct binding to myosin (3, 4), and its subsequent removal establishes the catch state (5). The catch state is characterized by a marked slowing of crossbridge cycling rate, measured as energy usage (6–8) and mechanical behavior such as force-velocity relations and force redevelopment after a quick release (9–12). Catch exemplifies the high economy of smooth muscle: the ability to maintain force with a low expenditure of energy. In the ABRM, rapid relaxation, or release of catch, occurs on stimulation of serotonergic nerves, a response that is mediated by an increase in cellular cAMP and the activation of protein kinase A (13, 14).

We have shown that the catch state is regulated by the cAMP-dependent phosphorylation of a high molecular mass (≈600 kDa) protein in the intact and permeabilized ABRM (15). Several lines of evidence support this conclusion. In permeabilized muscles, only the catch-regulating (CR) protein, to the exclusion of other candidate proteins such as paramyosin (16, 17), regulatory light chains of myosin (18–20), and myosin heavy chain (21) undergoes a significant increase in phosphorylation that corresponds to the cAMP-induced release of catch. Inhibition of protein kinase A prevents both the cAMP-induced phosphorylation of the CR protein and the release of catch. cAMP-dependent thiophosphorylation of the CR protein before activation of the muscle prevents catch. During activation of intact muscles with acetylcholine, the CR protein undergoes dephosphorylation, presumably as a result of the action of a calcium-activated phosphatase (22). The release of catch by serotonin is associated with phosphorylation of the CR protein. The degree of catch that is attained is tightly linked to the previous history of the muscle and the dephosphorylation of the CR protein that occurs on reactivation of the muscle. In the present study, we show that the high molecular weight protein whose phosphorylation state regulates catch is structurally related to twitchin, and that its phosphorylation state plays an important role in modulation of force output and velocity of shortening under conditions not historically associated with the catch state.

MATERIALS AND METHODS

Muscle Preparation. The ABRMs were isolated from fresh *M. edulis* (Kip's Seafood, Cushing, Maine) as previously described (15). Muscle bundles 0.2–0.4 mm wide were permeabilized in 1% Triton X-100 in rigor solution for 30 min and then rinsed in rigor solution before additional experimental manipulation. All experiments were done at 20°C. Measurements of force production were made at Lo (the optimum length for force production) as described previously (15). Unloaded shortening velocity (Vo) was determined according to the method of Edman (23).

Solutions. Relaxing and activating solutions for permeabilized muscles contained: 1 mM Mg ATP; 0.5, 5, or 20 mM EGTA; 3 mM free Mg^{2+}; 0.5 mM leupeptin; 1 mM DTT; 5 mM Pi; 30 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]. Ionic strength was maintained at 202 mM with 1,6-diaminohexane-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: $[Ca^{2+}]$, Ca^{2+} concentration; ABRM, anterior byssus retractor muscle; CR, catch-regulating; Vo, unloaded shortening velocity.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB008202).

†To whom reprint requests should be addressed at: Department of Physiology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107. e-mail: siegmanm@jeflin.tju.edu.

N,*N*,*N*′,*N*′-tetraacetic acid, and the pH was 6.8. The pCa of the relaxing solutions with no added calcium were considered to be pCa>8. In the activating solutions, the total EGTA was kept at 5 mM, and pCa was adjusted by the addition of CaEGTA. For the production of catch, relaxing solution containing 20 mM EGTA was used. Rigor solution was similar to relaxing solution but contained no ATP, and the EGTA concentration was 2 mM. Phosphorylation of proteins was measured by inclusion of 20 μCi/ml [γ-^{32}P]-ATP (DuPont/NEN).

Muscle Extraction and SDS/PAGE. Muscles rapidly frozen in liquid N_2 were pulverized with 0.5 N $HClO_4$. The protein precipitates were solubilized in SDS sample buffer, passed through a 0.4-μm filter, and subjected to SDS/PAGE (24) on gels containing various percentages of acrylamide (15). Gels stained with Coomassie blue were scanned with a laser densitometer (Personal Densitometer, Molecular Dynamics), and autoradiography was performed on dried gels with a storage phosphor screen (PhosphorImager, Molecular Dynamics).

Protein Preparation. Twitchin from the ABRM of *M. galloprovincialis L*. (Kesennuma Bay, Japan) was isolated by using the procedure of Vibert *et al.* (25) for mini-titin. Modifications were the use of liquid N_2 in which the ABRM sample was pulverized and chromatography using DEAE-5PW (Tosoh, Tokyo) and gel filtration on G3000 SWG (Tosoh). Purification was monitored by SDS/PAGE. The yield of purified protein from 10 g of ABRM was ≈2 mg.

The purified protein (≈0.5 μg) was phosphorylated *in vitro* in 0.3 ml of a solution containing: 0.2 mM ATP, 50 mM Tris, 50 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4 with 18 μCi γ^{32}P-ATP (DuPont/NEN), and 3.6 units of the catalytic subunit of protein kinase A (Sigma). In some cases, 1 μg of the peptide inhibitor of protein kinase A (Sigma) was included. After 15 min, the reaction was stopped with 10% trichloracetic acid, and the protein precipitate was subjected to SDS/PAGE.

cDNA Cloning. Total RNA was prepared from ABRM from *M. galloprovincialis* (26). First-strand DNA was synthesized by using a T-primed first-strand kit (Pharmacia). PCR amplification was performed by using a set of mixed primers containing *Bam*HI recognition sequences: the 5′ primer, 5′-dCCGGATCCTA(C/T)GA(C/T)AA(A/G)TA(C/T)TA(C/T)CA(C/T)G-3′, was designed from a N-terminal amino acid sequence of a peptide from CR protein [obtained after lysyl endopeptidase (Wako Biochemicals, Osaka) proteolysis], and the 3′ primer, 5′-dACGGATCC GC(C/T)TC(C/T)TTIC(G/T)(A/G)TC(A/G)AA(A/G)TA-3′, was based on the sequence of *Aplysia* twitchin (ref. 27; underlined in Fig. 2). PCR was carried out by using forward and reverse primers (25 pmol) and 10 ng of the first-strand cDNA template. Denaturation was carried out at 94°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 2 min. The cycle was repeated 30 times. The DNA fragment amplified by PCR was digested with *Bam*HI, and digests were subcloned into a plasmid vector, pBluescript II. Sequencing was performed by using the Dye Deoxy terminator cycle sequencing kits with a DNA sequencer model 373S (Perkin–Elmer).

Production of Antibodies. Rabbit antibodies were generated to the peptide 291 to 314 of the *Mytilus* sequence (see Fig. 2) with an additional N-terminal Cys. A keyhole limpet hemocyanin conjugate was used for injection. The antibody was affinity purified by using the above peptide attached to Sulfolink coupling gel (Pierce) following the manufacturer's instructions.

Statistics. Data are reported as means ± SEM. In comparisons, $P < 0.05$ is considered significant.

RESULTS

The Release of Catch and its Relationship to Phosphorylation of a High Molecular Weight Protein. A useful model for the study of the regulation of catch is the permeabilized ABRM of *M. edulis*. The characteristics of this model and the features it shares with the intact muscle have been described (15). The experimental paradigm for producing and releasing catch in permeabilized ABRM shown in Fig. 1 forms the framework for the current study. The typical catch response of the permeabilized muscle is shown in Fig. 1*A*. The muscle is maximally activated in pCa 5 and then washed in relaxing solution (pCa >8) containing 20 mM EGTA to rapidly remove calcium. The removal of calcium results in a decay of force, consisting of an initial rapid component during which the muscle relaxes by about 25% in 30 sec, followed by a very slow decay of force lasting many minutes (15). Force recovery after a quick release (5% Lo) imposed at this time is minimal compared with a similar perturbation imposed during activation in pCa 5, reflecting a slow crossbridge cycling rate. This is catch. On addition of cAMP, the muscle relaxes rapidly. SDS/PAGE of proteins from muscles so treated shows that only a high molecular weight, CR protein becomes phosphorylated during the cAMP-mediated release of catch. When protein kinase A is inhibited with a synthetic peptide inhibitor (28), addition of cAMP has no effect on catch force, and the CR protein is not phosphorylated (Fig. 1*B*). Prevention of the phosphorylation of the CR protein also prevents the release of catch. These and other previously described experiments on both intact and permeabilized ABRM (15) strongly suggest that the catch state is regulated by the state of phosphorylation of the CR protein.

Identification of the CR Protein. The CR protein from fibers showed a mobility on SDS/PAGE that suggested a molecular mass of ≈600 kDa (15). Because twitchin (also known as mini-titin) has a similar molecular mass (29, 30), and it, too, can be phosphorylated by protein kinase A (31), we used a procedure designed for mini-titin to isolate and purify the CR protein. The mobility of the purified protein was identical to the CR band (Coomassie blue-stained and PhosphorImage) derived from strips of ABRM (Fig. 1 *C–E*). The isolated protein was phosphorylated by the catalytic subunit of protein kinase A, and phosphorylation was inhibited by the protein kinase A peptide inhibitor (Fig. 1*F*), as also observed for the CR protein from muscle strips. The amino acid compositions were determined for the CR band isolated from SDS gels of muscle strips and for the isolated protein. These were similar to one another and also to that predicted from the cDNA sequence of *Caenorhabditis elegans* twitchin (data not shown) (29, 30). The amino acid composition of CR protein is also clearly distinct from paramyosin of *M. edulis*. For example, in CR protein, proline and glycine are 7.9 and 13.1 mol %, respectively, and are 1.5 and 4.8 mol % in paramyosin. This excludes the possibility that the CR protein is an aggregated form of paramyosin.

A derived amino acid sequence for a partial cDNA of the isolated protein is shown in Fig. 2 and compared with corresponding sequences of *Aplysia californica* and *C. elegans* twitchins. Over the indicated region of the molecule, the *Mytilus* protein was 57.7% and 77.4% identical to *C. elegans* and *Aplysia* twitchins, respectively. This sequence contains a putative kinase domain, with the conserved GXGXXG sequence (residues 35–40) that is involved in ATP binding at the N-terminal margin. At the C-terminal side of the kinase domain are the suggested calmodulin and Ca^{2+}/S100A1$_2$ binding sites of twitchin (32, 33). A similar sequence (underlined in Fig. 2) was found in the *Mytilus* sequence, and a synthetic peptide based on this sequence was used for the production of polyclonal antibodies. The affinity-purified antibody reacted with a band in the appropriate position for twitchin using a muscle homogenate (Fig. 1*G*), and also corresponded to the band phosphorylated in permeabilized muscle (Fig. 1*H*). The isolated protein crossreacted with the polyclonal antibody (data not shown).

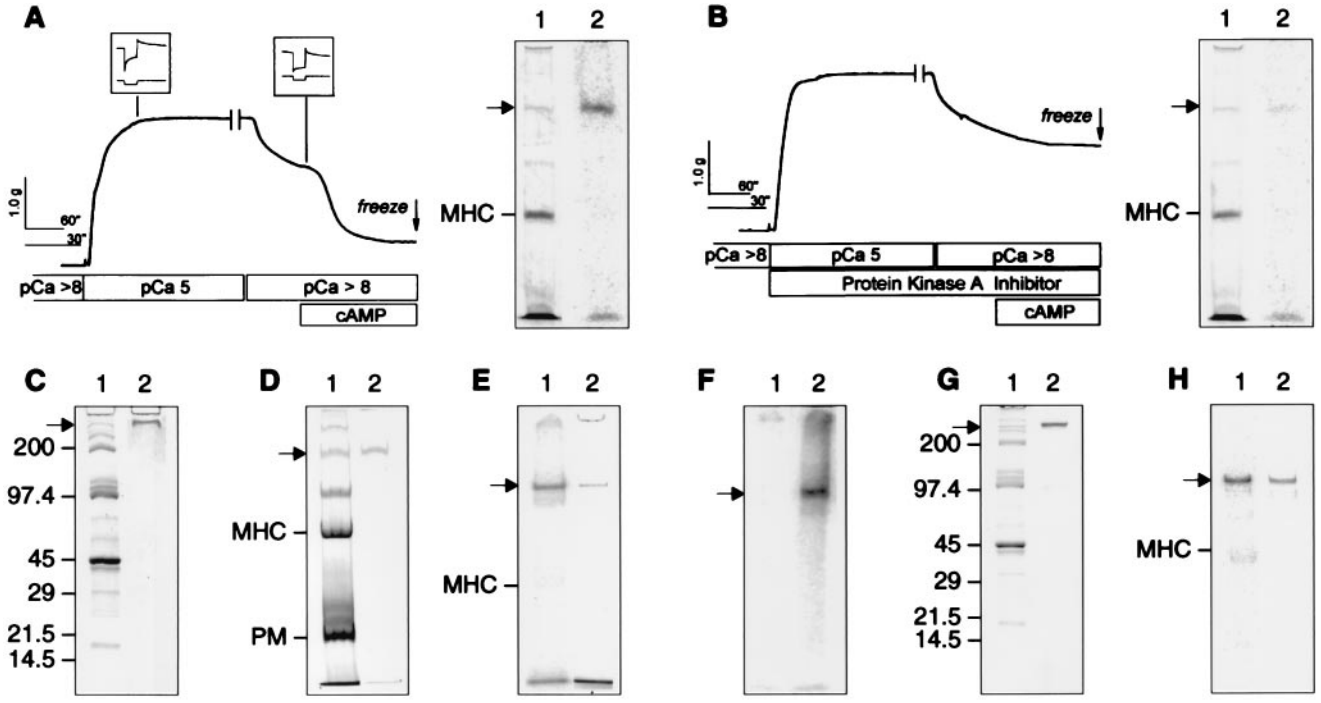

FIG. 1. Mechanical responses of the permeabilized ABRM during catch and the control of the release of catch by cAMP-dependent phosphorylation of a high molecular weight protein. (*A*) ABRM was activated in pCa 5 for 5 min, then washed in pCa >8 containing 20 mM EGTA. (*Inset*) Force responses (upper trace) to quick release in length of 5% Lo (lower trace), recorded for 10 sec after release. Addition of 100 μM cAMP causes rapid relaxation. γ^{32}P-ATP was included for the last 3.5 min of pCa 5 onward. Muscle was frozen 1 min after addition of cAMP. The break in the force trace indicates expansion of subsequent time scale. Also shown is Coomassie blue-stained 4% acrylamide gel (lane 1) containing total muscle protein, and its PhosphorImage (lane 2). (*B*) ABRM was subjected to same protocol as in *A*, except that peptide inhibitor of protein kinase A (10 μg/ml) was included. (*C–E*) 4–20%, 6%, and 4% acrylamide gels, respectively, containing the total protein from the ABRM (lane 1) and the protein purified as described in the text (lane 2). All are Coomassie blue-stained, except *E* (lane 1), which shows a PhosphorImage of an ABRM strip treated with cAMP and γ^{32}P-ATP. (*F*) A PhosphorImage of a 4% acrylamide gel containing the isolated protein treated with the catalytic subunit of protein kinase A and γ^{32}P-ATP in the presence (lane 1) and absence (lane 2) of a peptide inhibitor of the kinase. (*G*) A 4–20% acrylamide gradient gel of the total protein extract from the ABRM either stained with Coomassie blue (lane 1) or subjected to Western blotting with the antipeptide antibody (lane 2). (*H*) A PhosphorImage (lane 1) and a Western blot using the anti-peptide antibody (lane 2) from a 5% acrylamide gel containing protein from a permeabilized muscle, treated as described in *A*. MHC, myosin heavy chain; PM, paramyosin. → shows the position of the CR protein.

The data indicate that the CR protein is the *Mytilus* isoform of twitchin. Although there are some differences between the *C. elegans*, *Aplysia*, and *Mytilus* isoforms, the similarities are sufficient to merit this identification.

```
Mytilus        1   YDKYYHDLWKKYVPQPVSVRDANVSDYYEILEELGSGAFGVVHRCVEKATGRVFVAKFINTPYPLDKFAV
Aplysia       16   ...F.E.I.........E.KQGSCY...D.............................E............YT.
C.elegans   5124   ..N.VF.I..Q.Y....EIKHDH.L.H.D.H....T........VT.R...NN.A...VM..HES..ET.

Mytilus       71   KNEINVMNQCHHPKLLQLKDAFEDKYEMCLIFEFLAGGELFDRIAAEDYKMTECEVINYMRQICDGLKHM
Aplysia       86   ....SI...L......N.H.......D.V..L...S...............S.A........A.E.....
C.elegans   5194   RK..QT.SVLR..T.VN.H.....DN..VM.Y..MS.....EKV.D.HN..S.D.AVE....V.K..C..

Mytilus      141   HENSIVHLDVKPENVMCTTKNSNEVKMIDFGLATKLNPDEIVKVTTATAEFAAPEIVDREPVGFYTDMWA
Aplysia      156   ..H......I....I..E..KASS..I.....................................................
C.elegans   5264   ...NY....L....I.F...R...L.L.....TAH.D.KQS.....G........VAEGK...Y.....S

Mytilus      211   VGVLAYVLLSGLSPFAGEDDLETLANVQRCDWEFADDAFANISPEAKDFIRKLLIRQPQRRMTVHECLDH
Aplysia      226   I...G...................Q..K......DE...SSV........KN..QKE.RK.L...DA.E.
C.elegans   5334   ....S.I........G..N.D...R..KS...NMD.S..SG..EDG........LAD.NT...I.QA.E.

Mytilus      281   DWMK-GDLSGRTTRIPSSRYDSIRSKMRAKYADWPAPNPAIGRIANFGALRKNRPKDFSIFDS
Aplysia      296   P.L.-..H.NL.S.......NK..Q.IKE.........Q..........SS...H..QEYQ.Y..YFDRKEA
C.elegans   5404   P.LTP.NAP..DSQ......TK..DSIKT..DA..E.L.PL...S.YSS...H..QEY..R.A
```

FIG. 2. Primary structure of a portion of the CR protein derived from ABRM of *M. galloprovincialis*. Areas shown by the double underlines were used as primer regions for obtaining the cDNA sequence from which the amino acid sequence was derived. Also shown are the homologous regions of twitchin from *A. californica* (27) and *C. elegans* (29). The primer region of *Mytilus* twitchin as well as 8-Leu to 19-Val were determined by N-terminal amino acid sequencing, and the latter sequence was confirmed by the cDNA. The numbers represent residues from the N-terminal end of the fragment or molecule. The underlined peptide was synthesized and used to obtain a polyclonal antibody to the protein. Identical and gapped amino acids are shown by dots and dashes, respectively, for the best alignment of the three sequences.

Effect of Phosphorylation of Twitchin on Force Production. In the intact and permeabilized ABRM, the transition to catch after activation appears to rely on the reduction in intracellular [Ca^{2+}] to very low levels (2, 34). With the ability to manipulate the phosphorylation state of twitchin, it is possible to further probe its mechanism of action. Specifically, does the phosphorylation state of twitchin control force under conditions not usually considered to be associated with catch, e.g., suprabasal [Ca^{2+}]? Accordingly, force production was measured in muscles activated at specific [Ca^{2+}] and then treated with cAMP. The state of phosphorylation of twitchin and other proteins during the treatment was determined by inclusion of γ^{32}P-ATP in the bathing media.

At the outset, we examined the effect of cAMP on force production at pCa 5, the concentration at which maximal force was produced. As shown in Fig. 3*A*, cAMP caused the phosphorylation of twitchin yet had no significant effect on force production (force pCa 5 + cAMP : force pCa 5 = 0.997 ± 0.006, $n = 32$). At pCa 6, a concentration that produces about 45% maximal force, addition of cAMP caused phosphorylation of twitchin, but also significantly decreased force production (Fig. 3*B*). When the phosphorylation of twitchin is prevented by inhibition of protein kinase A (28), the decrease in force production on addition of cAMP in pCa 6 does not occur (Fig. 3*C*). To determine whether phosphorylation of any other protein underlies the reduction of force when cAMP is added at pCa 6, ABRM proteins were separated on gradient gels. The only protein showing any significant increase in phosphorylation on addition of cAMP is twitchin (Fig. 3*D*). Similar results (data not shown) were obtained when muscle proteins were solubilized directly into SDS buffer, thereby omitting treatment with 0.5 N $HClO_4$ and reducing the possibility of the loss of any acid-labile phosphorylation. These results strongly suggest that the reduction in force at the intermediate concentration of calcium is mediated by the phosphorylation of twitchin.

In light of the above results, it was of interest to determine the relationship between force production and [Ca^{2+}], and how this relationship was affected by cAMP. The results are shown in Fig. 4*A*. In these experiments, each muscle was treated, in a cumulative manner, with increasing concentrations of calcium. After a series of steady-state force measurements at a number of incrementing [Ca^{2+}] were completed, a transition was made to calcium solutions containing cAMP. The [Ca^{2+}] at which the transition was made differed for each muscle. At all submaximal [Ca^{2+}], the addition of cAMP caused the force to decline to some steady level, after which the muscle was bathed in further incrementing [Ca^{2+}] solutions containing cAMP. Given that the force at pCa 5 was unchanged by the addition of cAMP, the responses for each muscle were normalized to the force in pCa 5 + cAMP. At all concentrations tested, with the exception of pCa 5.3 and pCa 5, phosphorylation of twitchin significantly decreased force production. From calculation of A_{50} values (35), the [Ca^{2+}] that produced a half-maximal effect increased significantly from pCa 5.88 ± 0.04 to pCa 5.61 ± 0.02. That is, almost twice the [Ca^{2+}] (2.5 vs. 1.3 μM) is required for half-maximal force output when twitchin is phosphorylated. At the [Ca^{2+}] of 1.3 μM, twitchin phosphorylation decreased force from 50% to only ≈17% maximum force. These results clearly show that twitchin phosphorylation controls the calcium sensitivity of force output in the ABRM.

Vo at Intermediate [Ca^{2+}]. The ability of cAMP to reduce force in muscles activated at intermediate [Ca^{2+}] suggested

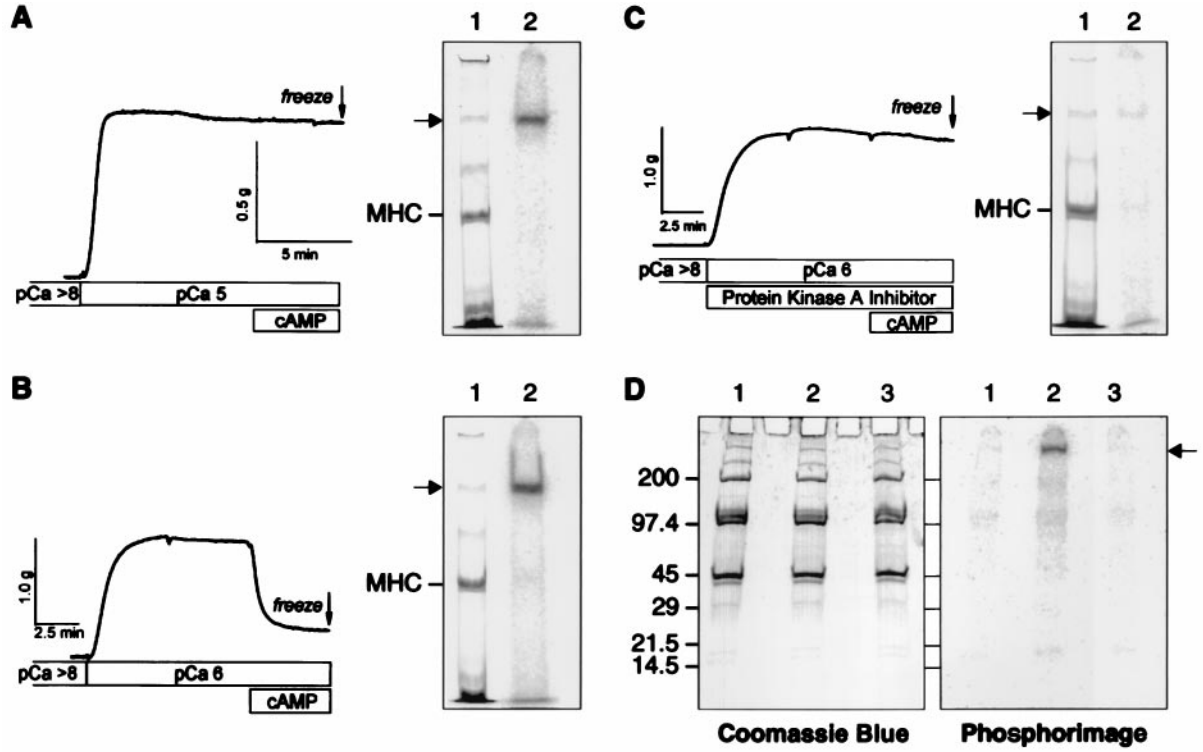

FIG. 3. The effect of cAMP on the mechanical responses of permeabilized ABRM during activation at high (pCa 5) and intermediate concentrations (pCa 6) of calcium. Different muscle strips were used for each treatment. (*A*) Muscle was activated in pCa 5 and then 100 μM cAMP was added and the muscle was frozen. γ^{32}P-ATP was included from 1.5 min in activating solution on. Also shown is Coomassie blue-stained 4% acrylamide gel (lane 1) and corresponding PhosphorImage (lane 2) prepared from frozen muscle. (*B*) Same protocol as in *A* except that this muscle was activated in pCa 6. (*C*) Same protocol as in *B* except that bathing media for this muscle included peptide inhibitor (10 μg/ml) of protein kinase A. (*D*) 4–20% gradient gel and corresponding PhosphorImage. Lane 1, from control muscle frozen 10 min after activation in pCa 6; lane 2, from muscle activated in pCa 6 for 10 min and treated with cAMP for 3 min; and lane 3, from muscle treated as in *B* but in presence of peptide inhibitor of protein kinase A. MHC, myosin heavy chain. → shows the position of the CR protein.

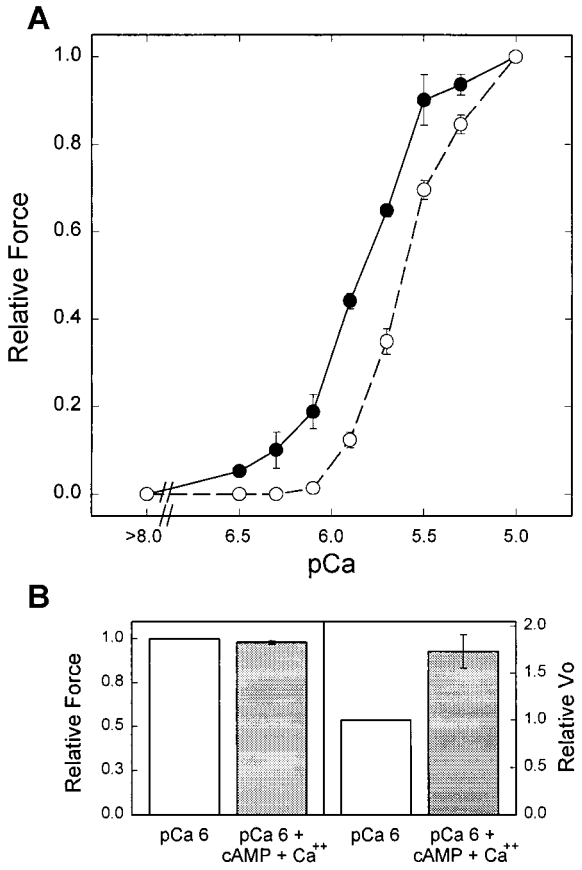

FIG. 4. (*A*) Effect of cAMP on the relationship between isometric force and $[Ca^{2+}]$ in permeabilized ABRM. ●, control; ○, + cAMP. See text for details of protocol. Force responses were normalized to the maximum force at pCa 5 + cAMP. (*B*) Effect of cAMP on Vo at pCa 6 in permeabilized ABRM. Vo was determined during steady-state force maintenance after activation in pCa 6. The muscle then was treated with cAMP, which caused partial relaxation. Calcium was added to the bathing medium until the force matched the initial force in pCa 6 alone, and Vo was determined again. The force responses and Vo were normalized to the initial responses in pCa 6 for each muscle (n = 15).

that some of this force was caused by the catch state. If so, one would expect that phosphorylation of twitchin would lead to the removal of any resistance to shortening presented by linkages in catch that probably would not cycle (if at all) as fast as activated myosin crossbridges. Accordingly, Vo was determined by using several step-releases ranging from 3% to 8% Lo at pCa 6. Muscles then were treated with cAMP for 3 min, after which calcium was increased until the force produced equalled the initial force at pCa 6, and Vo was again determined. Vo for muscles activated at pCa 6 was 0.018 ± 0.004 Lo/sec (n = 8). At constant force production, a significant 1.75-fold increase in Vo occurred at the higher calcium concentration in the presence of cAMP.

DISCUSSION

We recently have reported that catch in invertebrate smooth muscle is controlled by the phosphorylation of a high molecular mass (≈600 kDa) protein (15). The major findings in the present study are that the protein is identified as twitchin, and that its state of phosphorylation also controls force production by modulating calcium sensitivity under conditions not historically considered to be associated with catch.

Twitchin is a member of a family of giant protein kinases, which also includes projectin and titin, having molecular masses from 700 to >3,000 kDa. Twitchin, the protein encoded by the gene *unc-22* in *C. elegans*, gained its name because animals lacking *unc-22* showed a nearly constant twitching rather than prolonged undulating contractions of the body muscles (36). Immunolocalization studies revealed that twitchin is associated with the A band of nematode striated muscle (37), molluscan fast striated muscle, and thick filaments of molluscan smooth and catch muscles (25). The sequence of twitchin includes multiple copies of a fibronectin type III-like domain and an Ig domain, in addition to a single protein kinase catalytic domain (29). The kinase activity of *Aplysia* and *C. elegans* twitchin is activated by $Ca^{2+}/S100A1_2$ (32). *Aplysia* twitchin also has been reported to bind calcium/calmodulin (32), although at a relatively low affinity (K_d = ≈70 nM) compared with myosin light chain kinase (K_d = ≈1 nM) (38).

The fact that twitchin contains a protein kinase domain raises the question of whether the A kinase-mediated phosphorylation of twitchin might exert its mechanical effect through a change in twitchin kinase activity, or through a direct effect of the phosphorylation of twitchin on contractile protein interactions. Even though twitchin can phosphorylate the regulatory light chain of myosin *in vitro* (39), we have found no change in ^{32}P labeling of the light chain in the transition to and from catch. We have found that there is a tight relationship between twitchin phosphorylation and the release of catch force (15), as well as a change in calcium sensitivity (Fig. 4*A*), and there is no evidence for any protein that would serve as a target for the putative twitchin kinase. For these reasons, we favor the view that phosphorylation of twitchin directly modulates contractile protein interaction. The amount of twitchin (molar ratio to myosin = 1:15) and a cAMP-induced phosphorylation of 0.5 mol/mol (15) suggests that a direct control of myosin crossbridge interaction would have to be highly cooperative. It is even conceivable that twitchin could serve as a mechanical link or control mechanical links other than myosin.

Catch has been thought to occur only when intracellular calcium returns to basal or near-basal concentrations after activation of the muscle (1, 5). At such low $[Ca^{2+}]$, high force is maintained with little or no crossbridge cycling, reflected by the absence of force recovery after quick release (9, 15) and very low rates of energy usage (7, 8). Further, conditions that lead to phosphorylation of twitchin, such as application of serotonin to intact muscles or cAMP to permeabilized muscles, cause rapid relaxation, or the "release" of catch. The results of the present study show that the control exerted by phosphorylation of twitchin on the catch state extends also to calcium-activated contractions, where calcium-driven crossbridge cycling and force production occur. The findings that twitchin phosphorylation partially relaxes muscles activated at intermediate calcium concentrations and that, at equivalent levels of activation, Vo of the muscle increases, suggests that the catch mechanism functions together with cycling crossbridges. The simplest interpretation of the velocity data is that under these conditions, the resistance to shortening that occurs during catch is removed by twitchin phosphorylation. The fact that the muscle does actively shorten even when catch is apparent at suprabasal $[Ca^{2+}]$, suggests that although catch linkages may slow shortening they do not totally prevent shortening resulting from cycling crossbridges. It is also possible that the specific properties of the structures responsible for the catch state, e.g., response to a quick release, may differ according to the $[Ca^{2+}]$. The absence of an effect of twitchin phosphorylation on force output at saturating $[Ca^{2+}]$ suggests that the force-generating capacity of a calcium-activated myosin crossbridge is not altered by twitchin phosphorylation. Rather, the effect of twitchin phosphorylation seems to be on force-maintaining structures that are present only when some myosin is not calcium-activated.

The genetic evidence from *C. elegans* suggests that twitchin may be implicated in the contractile cycle of striated muscle (36, 40). Twitchin is also present in a variety of non-catch muscles from *Aplysia* (31) and scallop (25). Probst *et al.* (31) have found a correlation between the cAMP-dependent phosphorylation of twitchin and the rate of relaxation of the non-catch accessory radula closer muscle of *Aplysia*. Thus, the effect of twitchin phosphorylation on the relaxation rate in both catch and non-catch muscles is strikingly similar. The maintenance of force, or, more specifically, the very slow relaxation that characterizes catch, and the relatively rapid rate of relaxation of phasic muscles may represent the extremes over which twitchin phosphorylation regulates contractile protein interaction. It is possible that what distinguishes "catch" and "non-catch" smooth and/or striated muscles from invertebrates may be the dynamics and/or control of twitchin phosphorylation. In addition to the effects described above of twitchin phosphorylation on the accessory radula closer muscle of *Aplysia*, this may account for the ability of serotonin to accelerate the relaxation rate of phasic muscles, such as molluscan heart (41, 42), and the radula muscle of *Buccinum undatum* (43). It remains to be seen whether the relationship between phosphorylation of twitchin and the ultimate regulation of contractile protein interaction is similar in all of these muscle types.

In their studies of mammalian smooth muscles, Murphy and colleagues (44) noted marked differences in shortening velocity at constant levels of force production. The similarity of this behavior to that of invertebrate smooth muscle during catch prompted the description of this behavior as "latch." Both catch and latch may involve slowly or noncycling force-generating crossbridges. We show that the catch state extends into calcium-activated contractions, and that, under these conditions, the presence or absence of catch results in marked differences in the velocity of shortening at equivalent forces. This makes the mechanical behavior of catch and latch even more similar. It is now clear that phosphorylation of twitchin plays an important role in the release of catch and the decrease in force, perhaps because of detachment of slowly or noncycling crossbridges. The counterpart of twitchin, if any, in releasing the latch state in mammalian smooth muscle remains to be identified.

We thank Susan U. Mooers, Chenqing Li, Srinivasa Narayan, Kiyoshi Kikuchi, Misako Nakaya, and Keiko Iwasaki for their technical assistance. This work was supported by National Institutes of Health Grant AR42758 (M.J.S.) and a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (S.W.).

1. Atsumi, S. & Sugi, H. (1976) *J. Physiol.* **257,** 549–560.
2. Ishii, N., Simpson, A. W. M. & Ashley, C. C. (1989) *Science* **243,** 1367–1368.
3. Kendrick-Jones, J., Lehman, W. & Szent-Gyorgyi, A. G. (1970) *J. Mol. Biol.* **54,** 313–326.
4. Block, S. M. (1996) *Cell* **87,** 151–157.
5. Baguet, F. (1973) *Eur. J. Physiol.* **340,** 19–34.
6. Bozler, E. (1930) *J. Physiol.* **69,** 443–462.
7. Nauss, K. M. & Davies, R. E. (1966) *Biochem. Z.* **345,** 173–187.
8. Baguet, F. & Gillis, J. M. (1968) *J. Physiol.* **198,** 127–143.
9. Jewell, B. R. (1959) *J. Physiol.* **149,** 154–177.
10. Lowy, J. & Millman, B. M. (1963) *Philos. Trans. R. Soc. London B* **246,** 105–148.
11. Tameyasu, T. & Sugi, H. (1976) *J. Exp. Biol.* **64,** 497–510.
12. Tsuchiya, T. & Takei, N. (1986) *Comp. Biochem. Physiol. A* **83,** 519–523.
13. Cole, R. A. & Twarog, B. M. (1972) *Comp. Biochem. Physiol. A* **43,** 321–336.
14. Achazi, R. K., Dolling, B. & Haakshorst, R. (1974) *Pflügers Arch.* **349,** 19–29.
15. Siegman, M. J., Mooers, S. U., Li, C., Narayan, S., Trinkle-Mulcahy, L., Watabe, S., Hartshorne, D. J. & Butler, T. M. (1997) *J. Muscle Res. Cell Motil.* **18,** 655–670.
16. Achazi, R. K. (1979) *Pflügers Arch.* **379,** 197–201.
17. Watabe, S., Tsuchiya, T. & Hartshorne, D. J. (1989) *Comp. Biochem. Physiol. B* **94,** 813–821.
18. Sohma, H., Yazawa, M. & Morita, F. (1985) *J. Biochem.* **98,** 569–572.
19. Sohma, H., Sasada, H., Inoue, K. & Morita, F. (1988) *J. Biochem.* **104,** 889–893.
20. Sohma, H., Inoue, K. & Morita, F. (1988) *J. Biochem.* **103,** 431–435.
21. Castellani, L. & Cohen, C. (1987) *Science* **235,** 334–337.
22. Castellani, L. & Cohen, C. (1992) *FEBS Lett.* **309,** 321–326.
23. Edman, K. A. P. (1979) *J. Physiol.* **291,** 143–159.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. Vibert, P., Edelstein, S. M., Castellani, L. & Elliott, B. W. (1993) *J. Muscle Res. Cell Motil.* **14,** 598–607.
26. Chomiczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
27. Heierhorst, J., Probst, W. C., Vilim, F. S., Buku, A. & Weiss, K. R. (1994) *J. Biol. Chem.* **269,** 21086–21093.
28. Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., VanPatten, S. M. & Walsh, D. A. (1986) *J. Biol. Chem.* **261,** 989–992.
29. Benian, G. M., Kiff, J. E., Neckelmann, N., Moerman, D. G. & Waterston, R. H. (1989) *Nature (London)* **342,** 45–50.
30. Benian, G. M., L'Hernault, S. W. & Morris, M. E. (1993) *Genetics* **134,** 1097–1104.
31. Probst, W. C., Cropper, E. C., Heierhorst, J., Hooper, S. L., Jaffe, H., Vilim, F., Beushausen, S., Kupfermann, I. & Weiss, K. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8487–8491.
32. Heierhorst, J., Tang, X. X., Lei, J. Y., Probst, W. C., Weiss, K. R., Kemp, B. E. & Benian, G. M. (1996) *Eur. J. Biochem.* **242,** 454–459.
33. Heierhorst, J., Kobe, B., Feil, S. C., Parker, M. W., Benian, G. M., Weiss, K. R. & Kemp, B. E. (1996) *Nature (London)* **380,** 636–639.
34. Baguet, F. & Marchand-Dumont, G. (1975) *Pflügers Arch.* **354,** 75–87.
35. Tallarida, R. J. & Murray, R. B. (1987) *Manual of Pharmacologic Calculations with Computer Programs* (Springer, New York).
36. Waterston, R. H., Thomson, T. N. & Brenner, S. (1980) *Dev. Biol.* **77,** 271–302.
37. Moerman, D. G., Benian, G. M., Barstead, R. J., Schriefer, L. A. & Waterston, R. H. (1988) *Genes Dev.* **2,** 93–105.
38. Gallagher, P. J., Herring, B. P. & Stull, J. T. (1997) *J. Muscle Res. Cell Motil.* **18,** 1–16.
39. Heierhorst, J., Probst, W. C., Kohanski, R. A., Buku, A. & Weiss, K. R. (1995) *Eur. J. Biochem.* **233,** 426–431.
40. Moerman, D. G., Plurad, S. & Waterston, R. H. (1982) *Cell* **29,** 773–781.
41. Erspamer, V. & Ghiretti, F. (1951) *J. Physiol.* **115,** 470–481.
42. Fange, R. (1955) *Experientia* **11,** 156–157.
43. Fange, R. & Mattisson, A. (1958) *Acta Zool.* **39,** 53–64.
44. Dillon, P. F., Aksoy, M. O., Driska, S. P. & Murphy, R. A. (1981) *Science* **211,** 495–497.