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Phosphorylation of a high molecular weight (~600 kDa) protein regulates catch in invertebrate smooth muscle

MARION J. SIEGMAN[1]*, SUSAN U. MOOERS[1], CHENQING LI[1], SRINIVASA NARAYAN[1], LAURA TRINKLE-MULCAHY[1], SHUGO WATABE[2], DAVID J. HARTSHORNE[3] and THOMAS M. BUTLER[1]

[1]*Department of Physiology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107, USA;* [2]*Laboratory of Aquatic Molecular Biology and Biotechnology, University of Tokyo, Tokyo 113, Japan, and* [3]*Muscle Biology Group, University of Arizona, Tuscon, AZ 85721, USA*

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Summary

A unique property of smooth muscle is its ability to maintain force with a very low expenditure of energy. This characteristic is highly expressed in molluscan smooth muscles, such as the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*, during a contractile state called 'catch'. Catch occurs following the initial activation of the muscle, and is characterized by prolonged force maintenance in the face of a low $[Ca^{2+}]_i$, high instantaneous stiffness, a very slow cross-bridge cycling rate, and low ATP usage. In the intact muscle, rapid relaxation (release of catch) is initiated by serotonin, and mediated by an increase in cAMP and activation of protein kinase A. We sought to determine which proteins undergo a change in phosphorylation on a time-course that corresponds to the release of catch in permeabilized ABRM. Only one protein consistently satisfied this criterion. This protein, having a molecular weight of ~600 kDa and a molar concentration about 30 times lower than the myosin heavy chain, showed an increase in phosphorylation during the release of catch. Under the mechanical conditions studied (rest, activation, catch, and release of catch), changes in phosphorylation of all other proteins, including myosin light chains, myosin heavy chain and paramyosin, are minimal compared with the cAMP-induced phosphorylation of the ~600 kDa protein. Under these conditions, somewhat less than one mole of phosphate is incorporated per mole of ~600 kDa protein. Inhibition of A kinase blocked both the cAMP-induced increase in phosphorylation of the protein and the release of catch. In addition, irreversible thiophosphorylation of the protein prevented the development of catch. In intact muscle, the degree of phosphorylation of the protein increases significantly when catch is released with serotonin. In muscles pre-treated with serotonin, a net dephosphorylation of the protein occurs when the muscle is subsequently put into catch. We conclude that the phosphorylation state of the ~600 kDa protein regulates catch.

Introduction

The hallmark of the contractile process in smooth muscle is its ability to maintain force with a very high economy, that is, a low expenditure of energy. This characteristic was noted in early oxygen consumption measurements on lamellibranch adductor muscle (Parnas, 1910) and during measurements of heat production during contraction of the pharynx of *Helix pomatia* (Bozler, 1930). More recent measurements of phosphagen breakdown (Nauss & Davies, 1966) and oxygen consumption (Baguet & Gillis, 1968) during contraction of the anterior byssus retractor muscle (ABRM) of the mussel *Mytilus edulis* confirm the high economy. Such molluscan smooth muscles are of particular interest, because of their ability to maintain force for many hours. Von Uexkull (1912) speculated that the muscle was caught in a contracted state by a ratchet mechanism which he called *Sperrung*, translated by Bayliss (1927) as ''catch''. Following the initial activation of contraction, the catch state ensues at a time when the intracellular calcium concentration has decreased to near-resting levels (Atsumi & Sugi, 1976; Ishii *et al*., 1989). Catch is characterized by a period of force maintenance, or more precisely, an extremely slow relaxation of force, in the face of a very low expenditure of energy (Nauss & Davies, 1966; Baguet & Gillis, 1968). The marked reduction in energy usage for maintaining a given force during catch suggests a marked slowing of the cross-bridge cycling rate. Numerous mechanical studies, based on force recovery following quick-releases, force–

*To whom correspondence should be addressed.

velocity relations and instantaneous stiffness, are consistent with the idea that during phasic contraction, cross-bridges cycle, but in catch, cross-bridges remain attached and do not cycle or cycle very slowly (Jewell, 1959; Lowy & Millman, 1963; Tameyasu & Sugi, 1976; Tsuchiya & Takei, 1986).

Catch muscles, such as the ABRM of *Mytilus edulis*, are innervated by cholinergic and serotonergic nerves. Cholinergic excitation produces contraction, a prerequisite for the catch state which then ensues. Serotonergic nerve stimulation then causes rapid relaxation. However, simultaneous stimulation of both nerve types results in a phasic contraction, that is, rapid force development and rapid relaxation. When catch is released by serotonin, there is no reduction of the phasic contractile response (Twarog, 1967). The release of catch by serotonin is mediated by an increase in cellular cAMP and the activation of the cAMP-dependent protein kinase (Cole & Twarog, 1972; Achazi *et al.*, 1974).

Catch muscles of molluscs contain actin filaments similar to those in other muscles (Chantler, 1983); however, the thick filaments are very long, having lengths of 70–100 μm. Each thick filament is surrounded by and able to interact with 17–20 actin filaments (Sobieszek, 1973). The thick filaments are composed of paramyosin, which forms a large-diameter core, and a monolayer of myosin, which covers the surface (Szent-Gyorgyi *et al.*, 1971). The function of paramyosin is not known, but its presence may explain, in part, the ability of catch muscles to support high force production of up to 15 $kg\,cm^{-2}$ (Marceau, 1909). The positive correlation between thick filament length and paramyosin content, and the proportionality between force production and thick filament length (Lowy *et al.*, 1964), support the idea that muscles with high paramyosin content may generate high levels of force. It is interesting that thick filaments of all invertebrate muscles contain some paramyosin, but only catch muscle filaments contain large amounts of this protein (Levine *et al.*, 1976).

Two main theories have been put forward to explain the basis of catch (reviewed by Ruegg, 1971). One is the ''paramyosin hypothesis'' (Ruegg, 1961), which assumes the operation of two functionally distinct linkage systems for contraction and for catch. The contractile links are between actin and myosin, but catch linkages involve paramyosin–paramyosin interactions. The ''linkage hypothesis'' (Lowy *et al.*, 1964) suggests that the links between actin and myosin filaments cycle rapidly during the early, phasic portion of contraction, but are either locked in the attached state or detach very slowly during the catch phase. Ultrastructural studies showing differences in the organization and distribution of thick filaments during phasic and catch contractions of ABRM (Hauck & Achazi, 1987) may have been influenced by fixation artefacts, and therefore have been inconclusive (Bennett & Elliott, 1989).

Phasic and catch contractions have also been studied in permeabilized muscles from ABRM. Calcium activates contraction by direct binding to myosin (Kendrick-Jones *et al.*, 1970; Szent-Gyorgyi & Chantler, 1994), and its removal establishes the catch state (Baguet, 1973). Catch can be released by the addition of cyclic AMP (cAMP) or by addition of the catalytic subunit of the cAMP-dependent kinase (Marchand-Dumont & Baguet, 1975; Cornelius, 1980, 1982; Pfitzer & Ruegg, 1982). The role of cAMP, with the activation of the cAMP-dependent kinase in the release of catch, is well-founded (Pfitzer & Ruegg, 1982; Castellani & Cohen, 1987; Watabe & Hartshorne, 1990), but the site(s) of phosphorylation is not known. Various studies have suggested that paramyosin (Achazi, 1979; Watabe *et al.*, 1989), myosin heavy chain (Castellani & Cohen, 1987), and the regulatory light chains of myosin (Sohma *et al.*, 1985, 1988a,b) can be phosphorylated. Calcineurin, a calcium-calmodulin regulated type 2B phosphatase (Castellani & Cohen, 1992), may play a role in the development and maintenance of catch force. It is not known, however, which protein(s) might be dephosphorylated.

The purpose of this study was to determine which proteins undergo a change in the degree of phosphorylation on a time-course that corresponds to the release of catch by serotonin in the intact muscle and by cAMP in the permeabilized ABRM. We have found that only one protein, having a molecular weight of about 600 kDa, which undergoes an increase in phosphorylation during the release of catch, consistently satisfied these criteria. Evidence from several lines of experimentation described here, on permeabilized and intact muscles, supports the view that phosphorylation and dephosphorylation of this high molecular weight protein regulates catch:

(1) inhibition of the cAMP-dependent protein kinase, which prevented the cAMP-induced increase in the phosphorylation of the protein, inhibited the cAMP-dependent release of catch;

(2) cAMP-dependent thiophosphorylation of the protein prevents catch;

(3) in the intact muscle, the degree of phosphorylation of the protein is low when the muscle is activated with acetylcholine (ACh) and put into catch, and a significant increase in phosphorylation of the protein occurs when catch is released by treatment with serotonin.

We conclude that in the ABRM of *Mytilus edulis*, the catch state is regulated by the cAMP-dependent

phosphorylation of this high molecular weight protein.

Materials and methods

Solutions

Artificial sea-water (ASW). 10 mM KCl; 50 mM $MgCl_2$; 10 mM $CaCl_2$; 428 mM NaCl; 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES). pH was 7.4.

Relaxing and activating solutions for permeabilized muscles. 1 mM MgATP; 0.5 or 20 mM ethyleneglycol-bis (β-aminoethyl ether)-N′,N′-tetraacetic acid (EGTA); 3 mM free Mg^{2+}; 0.5 mM leupeptin; 1 mM dithiothreitol; 5 mM Pi; 30 mM piperazine-N,N′-bis[2-ethanesulphonic acid] (PIPES); ionic strength was maintained at 202 mM with 1,6-diaminohexane-N,N,N′,N′-tetraacetic acid (HDTA), 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, but pCa was adjusted by the addition 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. For thiophosphorylation, rigor solution containing 100 μM adenosine 5′-*O*-(γ-thiotriphosphate) (ATPγS) (Boehringer) was used. The synthetic peptide inhibitor of cAMP-dependent protein kinase (Cheng *et al*., 1986) was obtained from Sigma Chemical Co. and used at 10 μg ml^{-1}. [γ-^{32}P]ATP, [γ-^{33}P]ATP, [^{35}S]ATPγS and [^{32}P]-Pi were obtained from DuPont NEN.

Muscle preparation

Mytilus edulis was obtained from Anastasi's Fish Market, Philadelphia, within 36 hours of harvest. The mussels were housed in an aquarium containing aerated and recirculating seawater (Instant Ocean) at 4° C until sacrificed, a period usually no longer than three weeks. The shell was opened, the ABRMs were exposed, and the pedal ganglia were removed to prevent excitation of the muscles (Cole & Twarog, 1972). Muscle bundles 0.2–0.4 mm in width were isolated, mounted in stainless steel holders and incubated in aerated ASW at 20° C until use.

Intact muscle preparations were mounted on a myograph. For experiments on permeabilized muscles, the muscles were permeabilized by incubation in either 0.1% saponin or 1% Triton X-100 in rigor solution for 30 min, and then rinsed in rigor solution before further experimental manipulation. The responses after Triton X-100 permeabilization were similar to those we and others have obtained with saponin (Cornelius, 1980; Castellani & Cohen, 1987). All experiments were done at 20° C.

Mechanical measurements

For measurements of force production, muscle bundles 4–5 mm in length were mounted on a myograph as described previously (Siegman *et al.*, 1984). Muscle length was adjusted to slack length, *Ls*, taken as the length at which resting force is detectable during exposure to 50 μM serotonin (Cornelius & Lowy, 1978). Preliminary trials confirmed that in the ABRM, *Ls* coincides with *Lo*, the optimum length for active force production (Cornelius & Lowy, 1978). For the determination of force redevelopment following a quick-release, muscle bundles 3 mm in length and 0.2 mm wide were attached to the vane of a servomotor (Cambridge) and a force transducer (Akers 801). Quick-release steps of 5% muscle length were made from *Lo* in 0.5 ms, and the subsequent force responses measured for 10 s before restretch to *Lo*.

Muscle extraction and PAGE

Frozen muscles were pulverized and extracted with 0.5 N $HClO_4$. When appropriate, the acid extract was adjusted to pH 7.4 with KOH and subjected to high-performance liquid chromatography (HPLC) (Butler *et al*., 1990) for separation of nucleotides. The protein precipitates were solubilized in SDS sample buffer, filtered through a 0.4 μm filter and subjected to SDS–PAGE (Laemmli, 1970) on gels containing various percentages of acrylamide. In some cases, 4–20% or 4–15% acrylamide gradient gels (Bio Rad) were used. Laser densitometry (Personal Densitometer, Molecular Dynamics) was performed on gels in which Coomassie Blue staining was quantitated, and quantitative autoradiography was performed on dried gels with a storage phosphor screen (PhosphorImager, Molecular Dynamics). In some cases, the phosphorylated protein was extracted from the gel and ^{32}P dpm quantitated using a previously described procedure (Butler *et al*., 1994). When required, proteins were blotted on to polyvinylidene difluoride (PVDF) membranes, and enhanced chemiluminescence detection of antibody binding was performed using a kit (Amersham Life Science, Inc.). The monoclonal antibody to chicken-breast nebulin was obtained from Sigma Chemical Co.

Caged cAMP and caged ^{33}P-ATP experiments

The synthesis of the 1-(2-nitrophenyl)ethyl P^3 ester of γ^{33}P-ATP (caged ^{33}P-ATP) was performed using a modification (Vyas *et al*., 1992) of the method described by Walker and coworkers (1988). The specific activity of the caged ^{33}P-ATP was 5000–10 000 Ci per mole. The P-1-(2-nitrophenyl)-ethyl ester of cAMP (caged cAMP) was purchased from Calbiochem. The apparatus for flash photolysis and rapid freezing, and its use, have been described (Butler *et al*., 1994; Vyas *et al*., 1992). In these experiments, the solutions contained 0.5 mM ATP, 20 mM phosphocreatine, 1 mg ml^{-1} creatine kinase, 0.034 mM caged ATP (~0.12 mCi per ml caged γ^{33}P-ATP) and 20 mM total EGTA. When caged cAMP was used, it was at 5 μM.

^{32}P-ATP labelling of intact muscle

A group of six muscles was placed in 1.5 ml of ASW containing 50 μCi ^{32}P-Pi ml^{-1} (carrier-free). Incubation was either 18 h at 4° C or 5 h at 20° C. Following several washes in fresh ASW, the muscles were treated according to the specific experimental designs described in the Results section. Analysis of the 4% gels of muscle proteins included quantitation of the Coomassie Blue staining of the myosin heavy-chain band in each sample, and comparison with a myosin heavy-chain standard added to each

gel. Total numbers of counts in various bands were determined by volume integration of the phosphorimager image. In the experiments involving labelling for 5 h at 20° C, the specific activity of ^{32}P dpm in ATP was determined in each muscle extract. The optical density of the ATP from HPLC was compared with known standards and collection, and liquid scintillation counting of appropriate fractions gave ^{32}P dpm. In some samples, the fraction of the total ^{32}P dpm in ATP associated with the gamma phosphate was determined as described previously (Butler *et al.*, 1990). In 3–5 muscles of each of the experimental designs, the average fraction of the total ^{32}P-ATP dpm in the gamma phosphate of ATP was 0.53–0.56, with no significant differences between any of the designs.

Results

The standard protocols for the production of catch and the release of catch in intact muscles, and following their permeabilization, are shown in Figure 1. In Fig. 1A the intact muscle was treated with ACh for 1.5 min, which was then washed out with ASW. The inset traces show the force responses of the muscle to a quick-release of 5% *Lo* imposed during exposure to ACh and during force maintenance following its washout. Note that significant redevelopment of force occurs only when the quick-release is applied during the initial period of activation. The condition of high force with an extremely slow rate of decay following the removal of ACh, and little or no force redevelopment following quick-release, is catch. The prevailing force prior to the release is restored when the muscle is restretched to its initial length, showing a high stiffness in the face of little or no cross-bridge cycling during catch. These results are similar to those reported by Jewell (1959). Upon the addition of serotonin, the muscle relaxes rapidly, hence catch is released. The muscle was then permeabilized and activated in pCa 5 for 5 min (Fig. 1B). As in the intact muscle, force redevelopment occurred following a quick release. When washed in relaxing solution containing 20 mM EGTA (pCa >8) to remove calcium rapidly, there is typically a relatively slow decay of force, with little or no force redevelopment following a quick-release; this represents the catch state. In this example, after 1 min cAMP was added and the muscle relaxed rapidly. Qualitatively similar re-

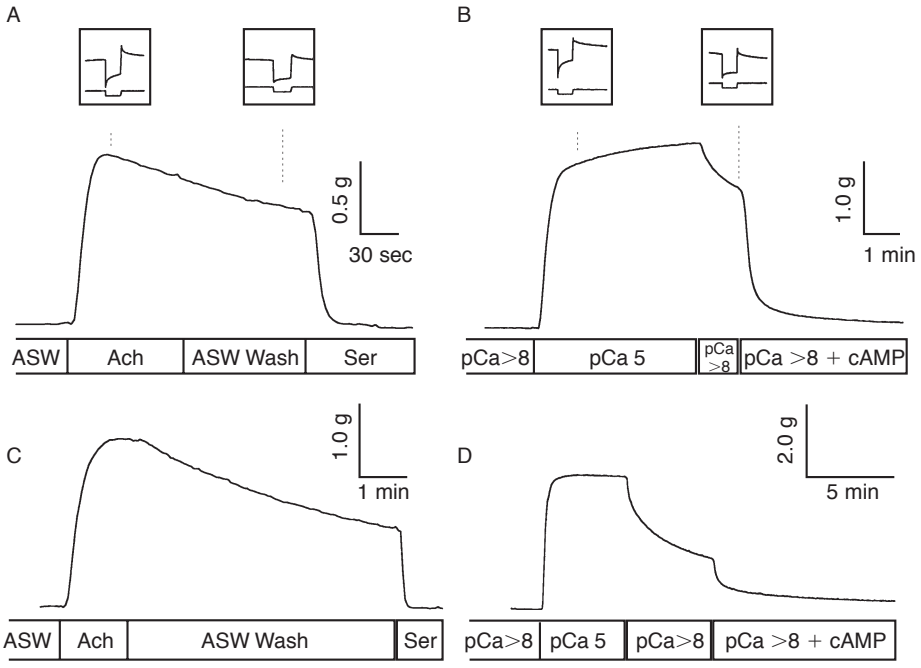

Fig. 1. Standard protocols for the production of catch and release of catch in intact ABRM and following permeabilization. (A) Intact muscle was activated with acetylcholine (50 μM) for 1.5 min, which was washed out with ASW for 1.5 min. Inset traces are force responses to quick-releases in length of 5% *Lo*, recorded for 10 s following the release. Note force recovery following quick-release during activation. The condition of high force maintenance, with little or no redevelopment of force following quick-release, is catch. Catch force is rapidly released upon addition of 10 μM serotonin. Muscle was then permeabilized (see text). (B) Permeabilized muscle was activated in pCa 5 for 5 min, then washed in pCa >8 containing 20 mM EGTA for 1 min. Note force recovery following quick-release during activation, but absence of recovery during wash in pCa >8. The latter is catch. The muscle rapidly relaxes upon addition of 10–100 μM cAMP. (C) and (D) The same protocols as in (A) and (B), except that the duration of catch is extended to 5 min in the intact and permeabilized muscles. Note the different calibration scales for the various panels.

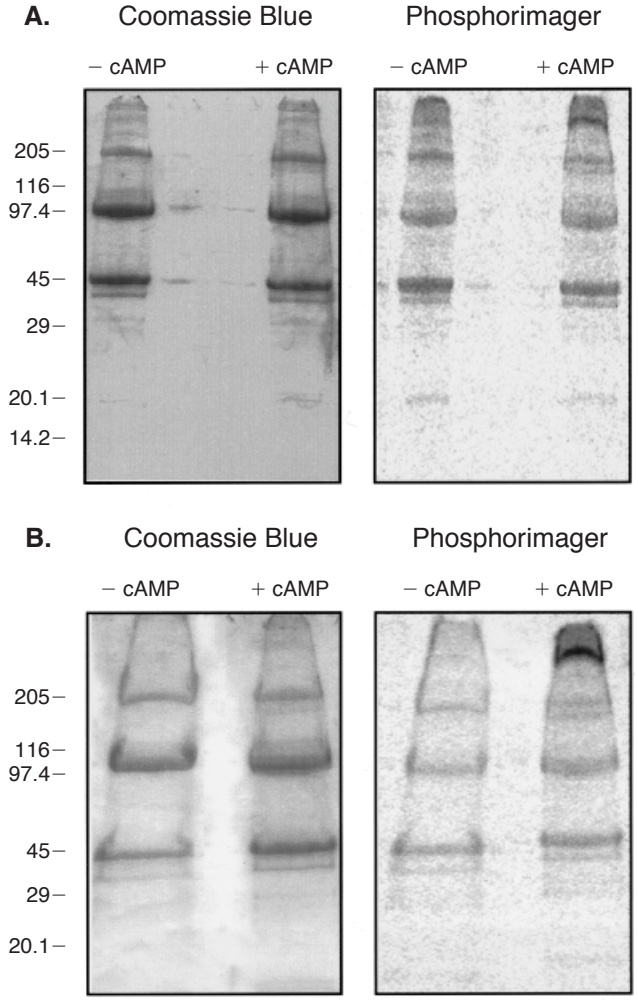

Fig. 2. Protein phosphorylation resulting from exposure to cAMP in permeabilized ABRM. (A) Use of caged cAMP. Coomassie Blue stained 4–20% acrylamide gradient gel and its autoradiogram obtained from the phosphorimager. Muscles were treated with caged ^{33}P-ATP either with (+cAMP) or without (−cAMP) caged cAMP, and frozen 10 s after photolysis. Experimental designs are described in the text. (B) Immersion in cAMP solution. Design similar to (A) except that the muscle (+cAMP) was rapidly and briefly (1 s) immersed in solution containing cAMP before photolysis of caged ^{33}P-ATP. The control (−cAMP) was identical except for the lack of immersion in cAMP.

sponses are obtained if the duration of catch is prolonged, as is shown in Figs 1C and 1D for an intact muscle and following its permeabilization, respectively. The time-course of catch force maintenance was followed in a group of permeabilized muscles ($n = 5$) for a period of 5 min. Within 15 s following transfer to relaxing solution (pCa > 8) containing 20 mM EGTA, the muscles showed no force redevelopment following a quick-release (data not shown). Compared with the force produced during activation in pCa 5, the mean catch force declined to $70 \pm 4\%$ at 30 s and changed fairly slowly thereafter ($62 \pm 5\%$ at 60 s, and $41 \pm 5\%$ at 300 s). In this study, however, the duration of catch was usually limited to 30 s or, in certain cases, 60 s, where the mean force was $75 \pm 0.9\%$ ($n = 103$) or $65 \pm 3\%$ ($n = 10$), respectively, of the force in pCa 5. These short time-periods were chosen not only because the catch state was established, but also to maximize catch force and subsequent relaxation responses.

Studies on permeabilized ABRM

The pivotal role of cAMP in the regulation of catch (Achazi *et al.*, 1974) led to the expectation that an A kinase-mediated phosphorylation of a protein or proteins would occur when catch is released. Such a regulatory phosphorylation must have a time-course which either precedes or at least is coincident with the decay of force. In order to determine which proteins change their state of phosphorylation under these conditions, the release of catch was initiated by the photolytic release of cAMP from caged cAMP, with a concomitant release of γ^{33}P-ATP from high specific activity caged γ^{33}P-ATP. In this experimental design, ATP (0.5 mM) was present throughout, but proteins could be labelled with ^{33}P only when cAMP was added and catch was being released. Specifically, skinned muscles were contracted in pCa 5 in the presence of both caged cAMP and caged ^{33}P-ATP. The muscle was then transferred to pCa >8 (20 mM EGTA) with the caged compounds for 30 s, followed by an ultra-violet flash which released both the radiolabelled ATP and cAMP. The muscle was frozen 10 s after the flash, and the muscle proteins were subjected to SDS–PAGE on gradient gels (4–20% acrylamide). Control muscles were treated identically, except that caged cAMP was not included. Figure 2A shows typical gels with autoradiograms from such an experiment. There was very little difference in the phosphorylation state of any proteins, except for a very high molecular weight protein, which was highly phosphorylated when cAMP was photolytically released.

The use of caged cAMP allows the precise control of the time of addition of cAMP; however, we noticed that its use resulted in some decay of force even before photolysis. We investigated this, and have come to the preliminary conclusion that caged cAMP partially activates the cAMP-dependent protein kinase in this skinned muscle system (data not shown). This result made the use of caged cAMP less than optimal, and instead the muscle was briefly (1 s) immersed in 100 μM cAMP followed by ultra-violet flash to release ^{33}P-ATP. Mechanical experiments showed that this was an effective way to release catch quickly. Figure 2B shows results from such an experiment in which the muscles were frozen 10 s

after the release of ^{33}P-ATP. There is a large cAMP-dependent phosphorylation of a high molecular weight protein with little, if any, cAMP-dependent change in the phosphorylation state of other proteins. If the cAMP is added 3 min before the release of ^{33}P-ATP, then there is no increase in the ^{33}P content of the high molecular weight protein when the muscles are frozen at 10 s after the flash (data not shown). Presumably, in this experimental design, the protein is maximally phosphorylated with unlabelled phosphate before the ^{33}P-ATP is available.

Figure 3 shows scans of the autoradiograms of gels from muscles frozen at various times after the addition of cAMP and photolytic release of ^{33}P-ATP. The large change in labelling of the high molecular weight protein is quite apparent. The total counts in each major protein band were determined, and the data are summarized in the inset of Fig. 3, together with a force trace showing the time-course of decay in force following addition of cAMP. The time-course of the increase in phosphorylation of the high molecular weight protein is coincident with the decrease in force associated with the release of catch. There is very little change in the labelling of proteins with molecular weights corresponding to actin, paramyosin, and myosin heavy chain. Also, the only protein to show a consistent increase in labelling upon addition of cAMP was the high molecular weight protein.

The cAMP-dependent phosphorylation of the high molecular weight protein is also apparent when the muscle proteins are run on low percentage (4%) acrylamide gels to increase the resolution of high molecular weight proteins (Fig. 4). In order to obtain an estimate of the apparent molecular weight of the phosphorylated protein, the mobility was compared with that of nebulin from chicken-breast muscle (Fig. 4B). The high molecular weight protein has a slightly higher mobility than nebulin in 4% acrylamide gels. Nebulin from chicken-breast muscle has a molecular weight of about 700 kDa (Kruger *et al.*, 1991), so ~600 kDa is a good estimate of the molecular weight of the protein which changes its state of phosphorylation when catch is released.

The main advantage that the use of caged ^{33}P-ATP affords in these experiments is the precise timing of phosphorylation associated with cAMP addition and relaxation. However, its use imposes certain limitations, such as a changing specific activity owing to the presence of an ATP-regenerating system, which precludes quantification of the amount of phosphate incorporated during this phosphorylation. Therefore, a simpler protocol was implemented. Muscles were incubated in solutions containing γ^{32}P-ATP under conditions that would allow for the specific activity of the nucleotide in the muscle to reach a steady state. In order to

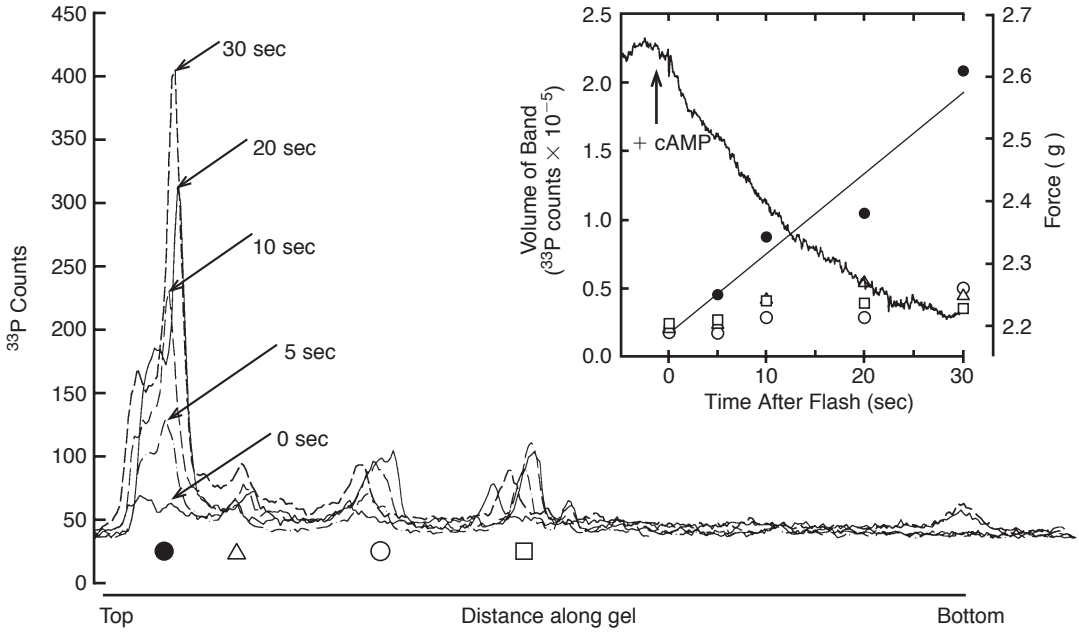

Fig. 3. Time-course of ^{33}P incorporation into proteins of permeabilized ABRM following cAMP treatment and photolysis of caged ^{33}P-ATP. Phosphorimager scans of 4–20% gradient gels containing proteins from muscles treated with cAMP as in Fig. 2B prior to photolysis of caged ^{33}P-ATP, and frozen at times indicated. Inset shows time-course of release of catch in relation to ^{33}P incorporation in protein bands, following cAMP treatment and photolysis of caged ^{33}P-ATP. Labelled proteins are shown by the following symbols: □, ~45 kDa; ○, ~100 kDa; △, ~200 kDa; ●, high molecular weight protein.

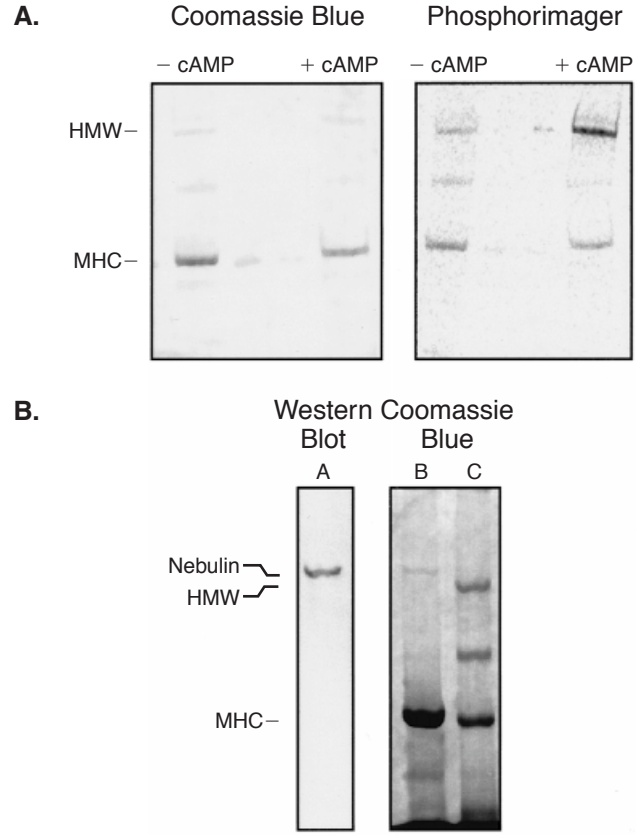

Fig. 4. Phosphorylation of a high molecular weight protein with treatment of permeabilized ABRM with cAMP. (A) Coomassie Blue-stained 4% acrylamide gel and its autoradiogram from the phosphorimager (experimental design as described in Fig. 2B). Muscles were either treated with cAMP (+cAMP) or not (−cAMP), and frozen 10 s after photolysis of caged ^{33}P-ATP. High molecular weight protein (HMW) and myosin heavy chain (MHC) are identified. (B) Comparison of the apparent molecular weight of HMW with nebulin on 4% acrylamide gels. Lane A is a Western blot of chicken-breast muscle stained with an antibody to nebulin, and B and C are the Coomassie Blue-stained lanes containing chicken-breast muscle and ABRM, respectively. Note that the high molecular weight protein has a somewhat higher mobility than nebulin.

quantify and compare the degree of phosphorylation during catch and following its release by cAMP treatment, the following protocol was used: skinned muscles were contracted in pCa 5 for 5 min and then transferred to a pCa >8 solution containing ^{32}P-ATP for 30 s, to establish catch. The muscle was then either (a) incubated in the same solution for additional 30 s and frozen, or (b) treated with a similar solution containing 100 μM cAMP for 30 s, to release catch, and frozen. Figure 5 shows gels and corresponding autoradiograms from this protocol. The scans of the autoradiograms show that by far the vast majority of the ^{32}P label is present in the high molecular weight protein following treatment of the muscle with cAMP. It is also clear that there is minimal radioactivity in myosin heavy chain and paramyosin. In comparing the phosphorimager scans in Fig. 4A and Fig. 5, it is apparent that the radioactive labelling of proteins, other than the high molecular weight protein, is decreased when ^{32}P-ATP is used, rather than ^{33}P-ATP derived from caged ^{33}P-ATP. This is observed in all muscles, whether or not they have been treated with cAMP. We have found a similar difference in overall labelling of proteins with the use of ^{32}P-ATP compared with caged ^{33}P-ATP in other experiments on mammalian smooth muscle (data not shown). We do not know the cause of this difference.

The above experiment utilizing ^{32}P-ATP was also used to obtain an estimate of the extent of phosphorylation of the high molecular weight protein. The amount of protein was estimated by comparing the Coomassie Blue staining of the high molecular weight protein to that of a myosin heavy-chain standard included on each gel, and the number of moles of phosphate incorporated was estimated from the dpm extracted from the gels and the measured specific activity of γ^{32}P-ATP. Assuming a molecular weight of 600 kDa, the phosphate incorporated into the high molecular weight protein is 0.53 ± 0.03 ($n = 3$) mole per mole of protein.

To test further the connection between the mechanical effect and phosphorylation of the high molecular weight protein, experiments were performed in which the extent of phosphorylation of the protein was manipulated. A synthetic peptide inhibitor of cAMP-dependent protein kinase (Cheng *et al.*, 1986) was used to inhibit the cAMP-induced increase in phosphorylation of the high molecular weight protein. The effectiveness of this inhibitor is demonstrated in the gels shown in Fig. 6. In the absence of the inhibitor, there is a large cAMP-mediated increase in phosphorylation, and in the presence of the inhibitor there is little phosphorylation. Typical mechanical responses of the muscle in the absence and presence of the inhibitor are shown in Fig. 7. In the initial control response with no added inhibitor (Fig. 7A), there is a large increase in the rate of decay of force upon addition of cAMP. In contrast, following reactivation in the presence of the inhibitor, there is a slow rate of decline of force when the muscle is transferred from pCa 5 to pCa >8, and no effect of cAMP. If the muscle is initially activated in the presence of the inhibitor, as shown in Fig. 7B, the slow force decay in pCa >8 is not affected by cAMP. After washout of the inhibitor, however, typical catch and cAMP-dependent release of catch responses occur, signifying that the effect of the inhibitor is reversible.

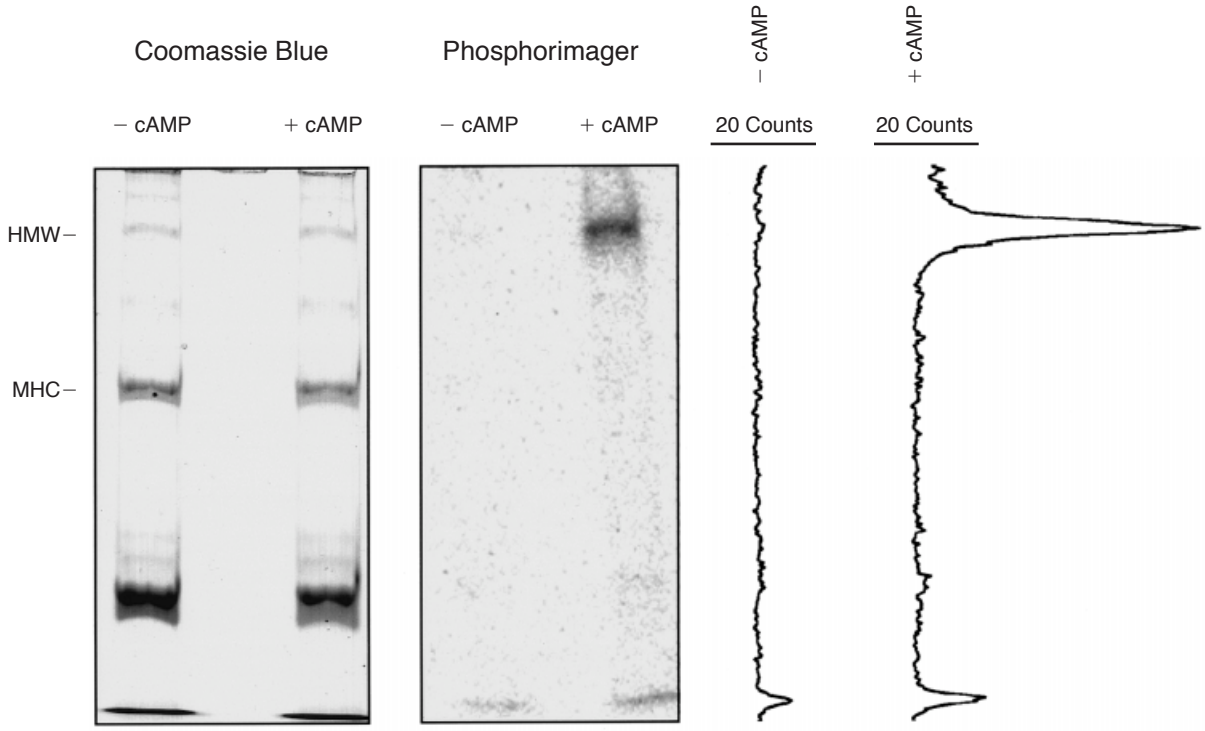

Fig. 5. Use of ^{32}P-ATP to demonstrate cAMP-induced phosphorylation of high molecular weight protein in permeabilized ABRM. Shown are Coomassie Blue-stained 4% acrylamide gels, the corresponding phosphorimager autoradiogram, and its scans for the two lanes. Upon transfer from pCa 5 to pCa >8, the muscle was incubated in ^{32}P-ATP for 30 s. cAMP was then added and the muscle frozen 30 s later (+cAMP). The control is similar except that no cAMP was added (−cAMP).

The high molecular weight protein can also be selectively thiophosphorylated with ATPγS in the presence of cAMP. Figure 8 shows gels of proteins from skinned muscles which were exposed to ^{35}S-ATPγS in the presence or absence of cAMP, and then incubated in a rigor solution for 9 min. In the former case, there was a large degree of thiophosphorylation of the high molecular weight protein, while in the latter case there was not. It is also noted that the large majority of the ^{35}S dpm was associated with the high molecular weight protein when cAMP was present. Therefore, the A kinase uses ATPγS to thiophosphorylate the protein, and the thiophosphorylated form of the protein is resistant to phosphatase activity, certainly for at least 9 min in rigor soution. The mechanical effect of pre-thiophosphorylation of the high molecular weight protein is also shown in Fig. 8. In muscles pre-treated with ATPγS in the presence of cAMP, the subsequent transition from pCa 5 to pCa >8 is associated with a very rapid decrease of force, and little or no effect of the addition of cAMP. This stands in contrast to responses of control muscles, which typically show catch when transferred to pCa >8, and rapid relaxation following the addition of cAMP.

All of the data presented so far suggest that there is long-term maintenance of catch force following the transition from pCa 5 to pCa >8, when the high molecular weight protein is not phosphorylated, and that its phosphorylation by A kinase can prevent the development of catch, and relax catch once it is developed. However, the possibility that other proteins may undergo changes in their state of phosphorylation under the same conditions could not be excluded. In order to investigate this further, an experiment was performed in which permeabilized muscles were continuously incubated with ^{32}P-ATP starting in an initial relaxing solution, and then frozen: *(A)* in relaxing solution; *(B)* in activating solution (pCa 5); *(C)* after 1 min in relaxing solution following pCa 5 (catch); and *(D)* treatment as in *(C)* except that relaxing solution contained cAMP (release of catch). Results from typical gels are shown in Fig. 9. By far, the major incorporation of ^{32}P into any protein in all of these experimental designs occurs in the high molecular weight protein that is phosphorylated in the presence of cAMP. There is some ^{32}P incorporation into protein in the 20 kDa region when the muscle is activated in pCa 5, and this persists with transfer to pCa > 8 either with or without cAMP.

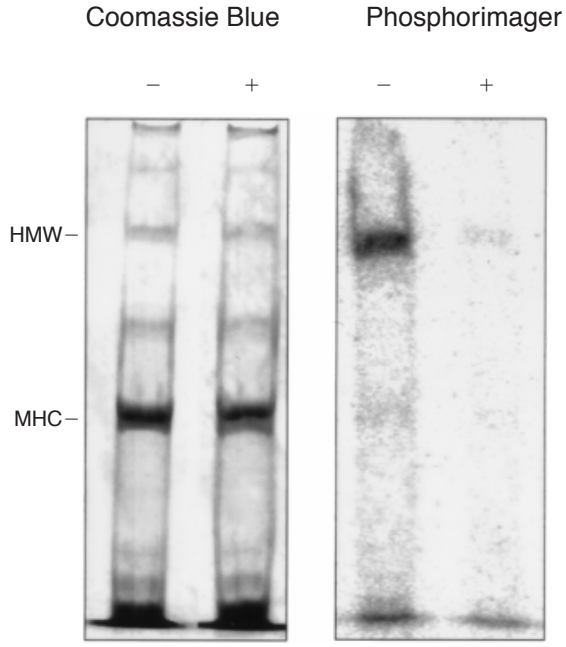

Fig. 6. Effect of peptide inhibitor of protein kinase A on cAMP-induced protein phosphorylation in permeabilized ABRM. Shown are Coomassie Blue-stained 4% acrylamide gels and corresponding autoradiograms from the phosphorimager. Muscles were frozen after being subjected to the standard catch protocol which included 5 min in pCa 5, 30 s in 0 Ca^{2+}, and then 1 min in 0 Ca^{2+} with cAMP. For the last 3.5 min in pCa 5, ^{32}P-ATP was added, and was included in all subsequent solutions. For control muscles (−), no peptide inhibitor was present; for test muscles (+), peptide inhibitor (10 μg ml^{-1}) was present in all solutions. The high molecular weight protein (HMW) and myosin heavy chain (MHC) are identified.

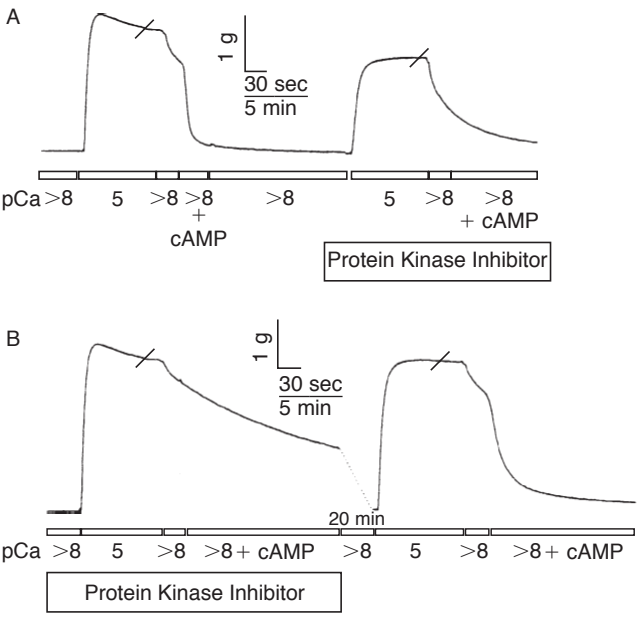

Fig. 7. Effect of peptide inhibitor of A kinase on mechanical responses of permeabilized ABRM. (A) Muscle was subjected to the standard catch protocol and subsequently washed extensively in relaxing solution to remove cAMP. The muscle was then treated with the protein kinase inhibitor peptide (10 μg ml^{-1}) and the catch protocol repeated. Note the decay of force and absence of cAMP effect in the presence of the inhibitor. (B) Muscle was initially treated with the inhibitor in relaxing solution and then during the standard catch protocol. Note the decay of force and absence of cAMP effect, as in (A). Following extensive washing in relaxing solution in the absence of the inhibitor, the muscle was subjected to the standard catch protocol. Note the restoration of the cAMP effect, indicating reversibility of the action of the inhibitor. Tick marks on force traces indicate expansion of the time base, as shown by calibrations.

The amount of ^{32}P in this band is only $13 \pm 2\%$ ($n = 8$) of that in the high molecular weight protein when catch is released. Also, there is no significant difference in the ^{32}P counts in the 20 kDa band in catch versus release of catch (ratio +cAMP: −cAMP $= 0.89 \pm 0.18$, $n = 7$). These results argue against the possibility that relaxation of catch results from a cAMP-mediated dephosphorylation of any protein whose phosphorylation state increases in high calcium or during the transition from high to low calcium.

Studies on intact ABRM

In these experiments, we sought to determine whether the phosphorylation state of the high molecular weight protein correlated with the mechanical state in the intact ABRM. Intact muscles were incubated in ASW containing ^{32}P-Pi at 4° C for 18 hours so that ATP in the muscle would be radioactively labelled. Following rewarming to 20° C and washing, muscles were frozen: *(A)* at rest in ASW; *(B)* after activation for 1.5 min in ACh; *(C)* after activation with ACh, followed by a 2 min ASW wash (catch); and *(D)* after treatment as in *(C)*, then 30 s in serotonin (release of catch). Muscle proteins were subjected to SDS–PAGE on 4% acrylamide gels, and it was found that there was radioactivity in both the high molecular weight protein (HMW) and myosin heavy chain (MHC) in muscles from each of the designs. In order to determine any change in labelling associated with the mechanical state of the muscle, the ^{32}P counts in the high molecular weight protein and in the myosin heavy chain were normalized to the quantity of Coomassie Blue staining of the myosin heavy chain in each lane. This allowed adjustment for differences in the amount of total muscle protein in each lane of the gel. Figure 10 shows the relative labelling of the two proteins in the various designs. There was a significant increase in labelling of the high molecular weight protein in the protocol for release of catch, compared with activa-

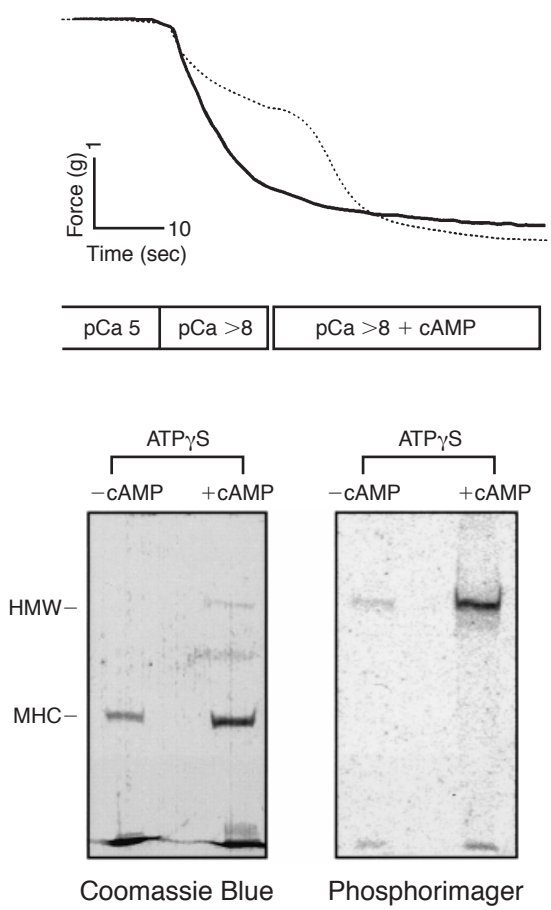

Fig. 8. Catch and release of catch in permeabilized ABRM and following cAMP-dependent thiophosphorylation of high molecular weight protein. Shown are Coomassie Blue-stained 4% acrylamide gels and phosphorimager autoradiograms of muscles treated with ^{35}S-ATPγS (100 μM) for 10 min in the presence (+cAMP) or absence of cAMP (−cAMP). This was followed by an incubation in rigor solution for 9 min in both designs. The high molecular weight protein (HMW) and myosin heavy chain (MHC) are indicated. Also shown is the mechanical effect of thiophosphorylation of the high molecular weight protein. Control muscles (dotted line) were activated in pCa 5 for 10 min, washed in relaxing solution (20 mM EGTA) to produce catch, then treated with 100 μM cAMP to release catch. Muscles initially treated with 100 μM ATPγS in the presence of 100 μM cAMP (solid line) were then subjected to the same procedures as control muscles. Note the absence of catch and cAMP effect.

tion with ACh, or subsequent catch. Although the labelling of the high molecular weight protein following the release of catch with serotonin was somewhat higher than at rest, the latter showed a relatively large variability and was not significantly different ($p > 0.05$). There is no significant change in the ^{32}P-phosphate content of the myosin heavy chain in any of the designs.

In a further series of experiments, the protocol was modified to include a shorter period of labelling (5 h) and the specific activity of the ATP was measured in extracts from each muscle, the better to normalize the data. Figure 11 shows a summary of the results including typical force responses from each of the designs. The shorter labelling period of these experiments resulted in much less labelling of the myosin heavy chain relative to the high molecular weight protein, and there was no change in myosin labelling associated with any of the mechanical states. For muscles in catch *(A)*, the labelling of the high molecular weight protein was about half that of the muscle that underwent release of catch *(B)*. Furthermore, the phosphorylation state of the high molecular weight protein was similar in resting muscles treated with serotonin *(C)* and those that underwent release from catch *(B)*. The last experiment in the series addressed the question of whether the protein, once phosphorylated at rest *(C)*, is subsequently dephosphorylated if the muscle is reactivated and goes into catch *(D)*. Indeed, there is a significant dephosphorylation of the high molecular weight protein when muscles undergo the transition from the resting state to activation and catch. Even though dephosphorylation occurs when the muscle goes into catch, the degree of phosphorylation in catch is significantly higher when the muscle has been pretreated with serotonin. It is interesting that catch force is maintained less well in this case (Fig. 11D versus A or B). In a separate experiment, the phosphorylation of the high molecular weight protein was determined in resting muscles after 30 s in serotonin, and compared with 30 s in serotonin followed by its washout in ASW for 30 min. The relative phosphorylations in these two designs were not significantly different (1.00 ± 0.18 *vs.* 1.11 ± 0.14 respectively, $n = 6$ in each). It appears that the serotonin-induced increase in phosphorylation of the high molecular weight protein is long-lived in the resting muscle, and that reactivation of the muscle in acetylcholine is required for dephosphorylation.

An estimate of the quantity of the high molecular weight protein relative to the myosin heavy chain in intact muscles can be obtained from comparisons of the Coomassie Blue staining of these proteins in gels. In 86 different intact muscles, the staining of the high molecular weight protein was $9.1 \pm 0.3\%$ of the myosin heavy chain. If these two proteins bind the stain to the same extent, then there is about ten times more myosin heavy chain on a weight basis; considering the difference in molecular weight (200 000 *vs.* ~600 000), there is about one mole of high molecular weight protein per 30 moles of myosin head in the intact ABRM.

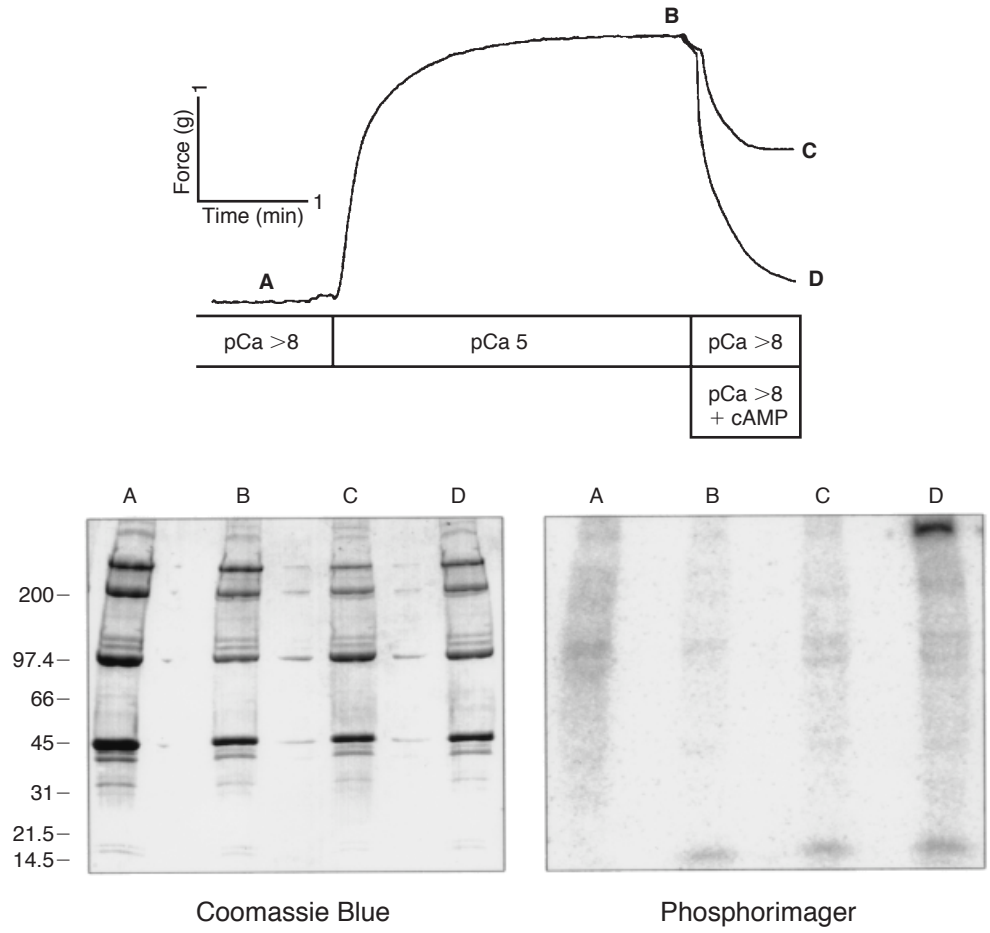

Fig. 9. Protein phosphorylation under different mechanical conditions in permeabilized ABRM. Muscles were continuously incubated with ^{32}P-ATP starting in an initial relaxing solution and then either frozen: (A) after 4 min in relaxing solution; (B) after 1 min in relaxing solution and 3 min in activation solution (pCa 5); (C) after treatment as in B, plus 1 min in relaxing solution (catch); and (D) after treatment as in B, plus 1 min in relaxing solution containing cAMP (release of catch). Shown are a Coomassie Blue-stained 4–15% acrylamide gradient gel and corresponding phosphorimager autoradiogram.

Discussion

Numerous mechanisms have been proposed to explain the regulation of contraction of mammalian smooth muscle, which differs from striated muscle in the independent control of force production and shortening velocity, exemplifed by its ability to vary the economy of force production. The ability to maintain force with a very low expenditure of energy is a characteristic of smooth muscle that has been conserved through evolution from molluscs (catch) to vertebrates (latch). The striking similarity of the catch state of molluscan smooth muscle and the latch state of mammalian smooth muscle prompted us to turn to the mollusc as an experimental model, which provides the advantage of neural control of relaxation as well as of activation. Although there is considerable evidence that serotonergic nerve stimulation causes rapid relaxation from catch by cAMP production and activation of a cAMP-dependent protein kinase, little is yet known regarding how these events are linked to the contractile process. The major new observation in this study is that the cAMP-dependent release of catch in ABRM is specifically associated with and regulated by the phosphorylation of a high molecular weight (~600 kDa) protein.

The high molecular weight protein, alone, consistently shows a change in state of phosphorylation during the release of catch in both intact (serotonin-treated) and permeabilized (cAMP-treated) muscles, and the time-course of phosphorylation is consistent with a role for this protein in regulating the release of catch. Under the same conditions, other proteins that have been suggested as candidates for regulation of catch, such as myosin heavy chain (Castellani & Cohen, 1987), paramyosin (Achazi, 1979; Watabe *et al.*, 1989), and myosin light chains

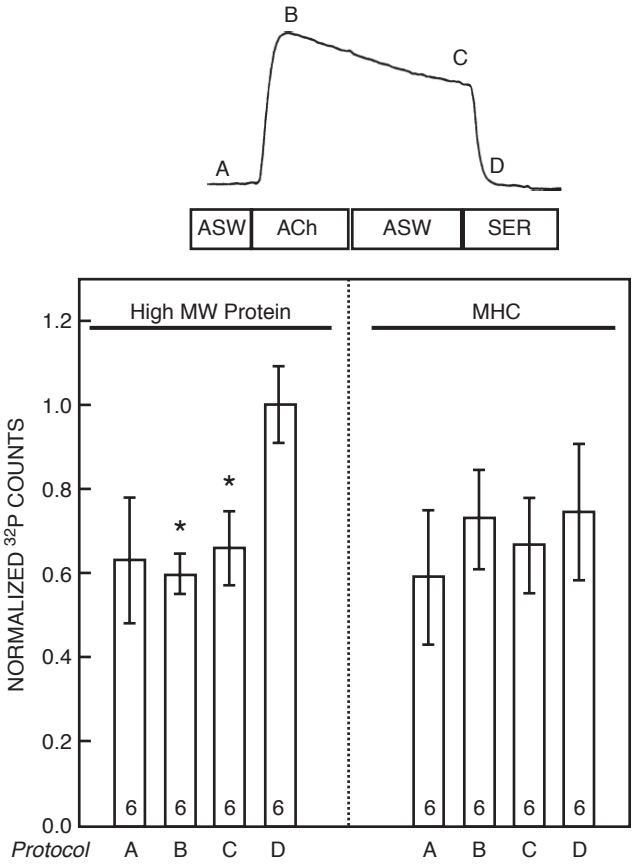

Fig. 10. ^{32}P content of the high molecular weight protein and myosin heavy chain (MHC) in intact muscles labelled with ^{32}P-Pi for 18 h at 4° C and under various mechanical conditions. Following rewarming to 20° C and washing, muscles were frozen: (A) at rest in artificial sea-water (ASW); (B) after 1.5 min in acetylcholine (50 μM); (C) after treatment as in B, followed by a 2 min ASW wash (catch); (D) after treatment as in C, but only 1.5 min ASW plus 30 s in 50 μM serotonin (release of catch). Phosphorylation was normalized to the staining of the myosin heavy chain and the mean of protocol D, and is reported relative to the mean value of protocol D. Data are shown as mean ± SEM with number of muscles in each protocol indicated. $^{*}p < 0.05$ compared with protocol D.

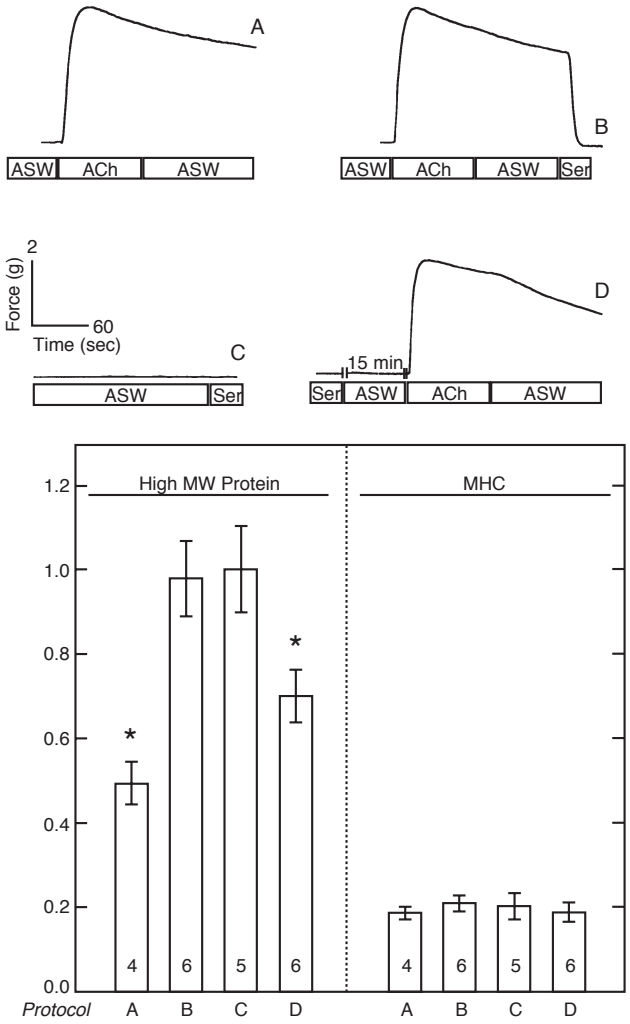

Fig. 11. ^{32}P content of the high molecular weight protein and myosin heavy chain (MHC) in intact ABRM labelled with ^{32}P-Pi for 5 h and subjected to various mechanical conditions. Phosphorylation was normalized to the staining of the myosin heavy chain in each lane and the specific activity of ^{32}P-ATP in each sample. Data are reported relative to the mean of protocol C. Protocols were as follows: (A) ACh (50 μM) for 1.5 min followed by ASW wash for 2 min; (B) treatment as in A, but only 1.5 min ASW wash, then serotonin (50 μM) for 30 s; (C) unstimulated muscle treated with serotonin (50 μM) in ASW for 30 s; and (D), treatment as in C, then 15 min ASW wash, followed by 1.5 min ACh and then ASW wash for 2 min. The force traces show typical responses of muscles for the protocols indicated. Data are shown as mean ± SEM with the number of muscles in each design indicated. $^{*}p < 0.05$ compared with all other protocols.

(Sohma *et al.*, 1985, 1988a,b; Sohma & Morita, 1986) do not show cAMP-dependent increases in phosphorylation. During the cAMP-dependent release of catch, the high molecular weight protein appears to incorporate about 0.5 mole of phosphate per mole of protein. The quantity of the high molecular weight protein in the muscle is estimated to be about 10% of the amount of myosin heavy chain.

A number of mechanical observations made previously by other investigators who studied catch in permeabilized ABRM have provided the basis for some of the experiments described here. The addition of cAMP (Cornelius, 1982) or the catalytic subunit of cAMP-dependent protein kinase (Pfitzer & Ruegg, 1982) under low calcium conditions (pCa >8) was shown to release catch. We have found an excellent temporal correlation between the cAMP-dependent increase in phosphorylation of the high molecular weight protein and the release of catch using a variety of experimental paradigms.

Although such a correlation is important, it alone is not sufficient to establish a causal relationship between these events. Therefore, it was important to determine the effects of manipulation of the state of phosphorylation of the protein on the mechanical behaviour of the muscle.

Castellani and Cohen (1987) showed that phosphatase inhibition with NaF, or the use of a non-hydrolyzable substrate such as ATPγS, prevents catch, but not the initial force development. These results suggested that the protein controlling catch could be thiophosphorylated, making it resistant to phosphatase activity, and that this manoeuvre might be used to set the degree of thiophosphorylation of the high molecular weight protein to a high level which could be maintained during cycles of contraction and relaxation in the permeabilized muscle. Indeed, when permeabilized muscles were incubated with ATPγS in the presence of cAMP, the high molecular weight protein was thiophosphorylated nearly to the exclusion of all others. When the protein was thiophosphorylated before activation, force production was unaffected, but catch was prevented and cAMP had no effect on relaxation. Castellani and Cohen (1992) also showed that poor catch force in permeabilized muscles could be improved by the addition of calcineurin, a calcium-calmodulin regulated type 2B phosphatase. Although we have not succeeded in confirming the latter results, we did observe a marked reduction of catch in intact muscles treated with cypermethrin, a calcineurin inhibitor (Enan & Matsumura, 1992; data not shown).

In another series of experiments, the cAMP-dependent kinase and the resulting phosphorylation of the high molecular weight protein was inhibited, using a peptide inhibitor of the enzyme. As before, force production was not significantly affected, nor was the degree of catch. However, the subsequent cAMP-dependent release of catch was blocked. These effects of inhibition of protein kinase A were fully reversible. All of these results on the correlation between phosphorylation of the protein and the release of catch provide strong evidence for the regulation of catch by the cAMP-dependent phosphorylation of the high molecular weight protein.

It might be argued that the release of catch may result from a cAMP-mediated dephosphorylation of a protein whose phosphorylation state increases when the muscle is activated in high calcium, or during the transition from high calcium to low calcium when catch is produced. Evidence from permeabilized muscles, however, showed only little incorporation of ^{32}P into any protein during these transitions. With activation, there was a small increase in phosphorylation in the region of the gel (~20 kDa) where myosin light chains would be expected to migrate. Although we have not done a detailed study of the extent of this phosphorylation, it is only about 10% of the cAMP-induced phosphorylation of the high molecular weight protein. This, together with the observations that the high molecular weight protein is phosphorylated to the extent of 0.5 mole per mole, and is present at a concentration 30 times lower than the myosin heavy chain, suggests that the change in phosphorylation of the light chain is less than 5%. *In vitro* studies have described the phosphorylation of the regulatory light chain from scallop catch muscle. Morita and colleagues (Sohma *et al*., 1985, 1988a,b) have suggested that such a phosphorylation releases the muscle from the catch state. However, we have found that the activation-induced increase in light chain phosphorylation did not decrease when the muscles were subsequently placed in low calcium, and, importantly, there was no significant change in labelling on the addition of cAMP and release of catch.

By far the major change in phosphorylation of any protein in any of the experiments occurred in the high molecular weight protein, which became highly phosphorylated during the cAMP-induced release of catch. The experimental results support the idea that the phosphorylation state of the high molecular weight protein directly controls whether catch is present or not at low calcium concentrations. In other words, the phosphorylation state of this protein determines whether the force generated by myosin during activation of the muscle persists when the calcium concentration is subsequently lowered. If the maintenance of catch force is through myosin cross-bridge interaction, then it appears that the phosphorylation state of the high molecular weight protein controls this interaction. This is remarkable considering that the protein is present at a molar concentration which is about 30 times lower than the myosin heavy chain.

It was important to determine whether the apparent regulatory role of the high molecular weight protein in the control of catch in permeabilized muscles also occurred in intact muscles. Using incubation of the intact muscle in ^{32}P inorganic phosphate to label ATP, the state of phosphorylation of muscle proteins could be followed during the various mechanical transitions and states. These experiments showed that there is a marked increase in the degree of phosphorylation of the high molecular weight protein during the release of catch induced by serotonin. In resting muscles treated with serotonin, there was no significant decrease in phosphorylation of the high molecular weight protein at 30 min after serotonin was washed out. Therefore the phosphorylation state of the protein is long-lived in the resting, unstimulated

muscle. On the other hand, there is a significant decrease in phosphorylation if the muscle which was pre-treated with serotonin was subsequently put into catch by stimulation with ACh. The cycle of phosphorylation and dephosphorylation of the high molecular weight protein can be summarized as follows: serotonin treatment results in phosphorylation of the high molecular weight protein (and release of catch), and the phosphorylation persists for many minutes in the resting muscle after serotonin is removed. There is a decrease in phosphorylation of the protein when the muscle is subsequently stimulated with ACh and goes into catch. The myosin heavy chain was also labelled with ^{32}P in these experiments, but, importantly, the degree of phosphorylation was not related to the mechanical state of the muscle.

A factor that complicates quantitation of the changes in phosphorylation of the high molecular weight protein during a cycle of contraction, catch and relaxation in the intact muscle, is that the protein always shows some degree of phosphorylation. There may be phosphorylation sites not involved in controlling catch, and the cAMP-induced changes in phosphorylation of the high molecular weight protein may be superimposed on this background phosphorylation. If this were the case, then in intact muscles, the difference in ^{32}P contents of the high molecular weight protein in catch (design A, Fig. 11) and the release of catch (design B, Fig. 11) may represent the maximum change in phosphorylation of the catch-regulating site. This would then be the equivalent of the 0.5 mole phosphorylation per mole of protein resulting from addition of cAMP to the permeabilized muscles.

There is considerable variability in the degree of phosphorylation of the high molecular weight protein in both the catch state and in unstimulated, ostensibly resting, intact muscles. The phosphorylation is significantly higher when the muscle is in catch following pre-treatment with serotonin than without such pre-treatment (see Fig. 10), and the variability 'at rest' is such that we could not show a significant increase in phosphorylation in comparing 'resting' with serotonin-treated muscles (see Fig. 9). Also the serotonin-induced phosphorylated state of the high molecular weight protein appears to be long-lived, even when serotonin is washed out. Therefore, the recent history of the muscle, together with the extent of its activation following a period of quiescence, may be important factors in determining the state of phosphorylation of the high molecular weight protein. These differences in extent of phosphorylation of the protein are likely to affect the mechanical properties of the muscle. The degree of catch is not as complete if the muscle has been treated with serotonin before acetylcholine activation (see Fig. 11), and Twarog and Cole (1972) noted that repeated activation with acetylcholine resulted in an accumulation of catch force. It is possible that the latter effect was due to a more complete dephosphorylation of the high molecular weight protein with repeated activation. It is conceivable, if not likely, that the ABRM functions at some intermediate state of catch *in vivo*, which would facilitate the tethering role of the byssus.

Collectively, the results suggest that during activation, a phosphatase causes a net dephosphorylation of the high molecular weight protein. The state of phosphorylation of the protein does not seem to affect force output in high calcium (see thiophosphorylation results), but dephosphorylation of the protein results in catch at low calcium concentrations. Catch is released when the high molecular weight protein is phosphorylated by cAMP-dependent protein kinase.

The identity of the protein is not yet known, but its large molecular weight makes it somewhat unusual. It is interesting that Achazi (1979) reported *in vitro* experiments in which a ~300 kDa unknown protein in molluscan muscle actomyosin was identified as a substrate for A kinase. We did not observe the phosphorylation of a 300 kDa protein with the release of catch, but given the difficulties in determining the molecular weight of such large proteins, it is possible that the protein that Achazi described is the high molecular weight protein observed in this study. Probst and coworkers (1994) have shown that a 750 kDa protein, which has considerable sequence homology to twitchin from *C. elegans* (Benian *et al.*, 1989), is a major substrate for A kinase in the accessory radula closer (ARC) muscle of the marine mollusc *Aplysia californa*. The rate of relaxation of the ARC muscle is correlated with the extent of phosphorylation of *Aplysia* twitchin. The authors show, however, that twitchin is also present in a variety of non-catch muscles, including gut, penis, heart and body-wall of *Aplysia*. This, and the fact that twitchin is present in both catch and non-catch muscles of the scallop (Vibert *et al.*, 1993), makes it difficult at this time to explain its role in non-catch contractions. It is possible that the regulatory role of the high molecular weight protein in catch represents a unique control of contractile protein interaction. However, the experiments reported here do not distinguish a mechanism for catch that is centred at the cross-bridge (''linkage'' hypothesis; Lowy *et al.*, 1964) from one involving structural protein interactions (''paramyosin'' hypothesis; Ruegg, 1961). Future studies will be required to address these questions.

In summary, the results presented here strongly suggest that the phosphorylation state of a

~600 kDa protein regulates catch in the ABRM of *Mytilus edulis*. Experiments on both permeabilized and intact muscles indicate that the following sequence of events occurs. When the muscle is stimulated with acetylcholine and the intracellular calcium concentration increases, the phosphorylation of the high molecular weight protein decreases, probably through the action of a calcium-activated phosphatase (Castellani & Cohen, 1992). The dephosphorylation of the protein does not alter force output as long as the calcium concentration is high, but when calcium returns to low levels (Ishii *et al*., 1989), continued force maintenance (catch) requires the protein to be dephosphorylated. Somehow, the interaction between contractile filaments resulting in force output is maintained at the low calcium concentration if the protein is dephosphorylated. When serotonin is added, there is an increase in cAMP, and the high molecular weight protein is phosphorylated by A kinase. This results in the loss of the catch state and a rapid relaxation of the muscle.

Acknowledgements

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