BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 4191–4199 4191
DOI: 10.1021/bi100143q

Myosin Kinase of Molluscan Smooth Muscle. Regulation by Binding of Calcium to the Substrate and Inhibition of Myorod and Twitchin Phosphorylation by Myosin

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Received January 29, 2010; Revised Manuscript Received April 13, 2010

ABSTRACT: Major contractile proteins were purified from relaxed actomyosin extracted from molluscan catch muscle myofibrils using ammonium sulfate fractionation and divalent cation precipitation. A fraction of this actomyosin was precipitated and purified as a supramolecular complex composed of twitchin (TW), myosin (MY), and myorod (MR). Another TW−MR complex was obtained via the removal of myosin. These supramolecular complexes and filaments assembled from purified myosin contained an endogenous protein kinase that phosphorylated myosin and myorod. Significantly, the activity of this novel myosin-associated (MA) kinase was inhibited at calcium concentrations of $>0.1\ \mu$M. After partial purification of the kinase, we established that the inhibition resulted from binding of calcium to the substrate (myosin) and not from the binding to the enzyme (kinase). No inhibition was observed when myorod was used as a substrate, although the latter is identical to the rod portion of myosin lacking the head domains. Phosphorylation sites of myorod were identified, three at the C-terminal tip and three at the N-terminal domain. In the presence of calcium, addition of myosin to the TW−MR complex resulted in inhibition of this phosphorylation, while in the absence of myosin, this inhibition was negligible. Added myosin also inhibited phosphorylation of twitchin by PKA-like kinase, the latter also present in the complex. The opposite was true with the TW−MY−MR complex; that is, phosphorylation of myosin was inhibited by twitchin and/or myorod. Thus, in parallel to the well-established direct activation by calcium, molluscan catch muscle myosin also regulated its own phosphorylation. Therefore, in addition to the established phosphorylation of twitchin by PKA-like kinase, phosphorylation of myosin and myorod by myosin-associated kinase appears to play an important role in the development of the catch state.

In muscle contraction, the release of calcium into cytoplasm is sensed by regulatory proteins and initiates contraction. In vertebrate skeletal muscle, these proteins form a regulatory complex composed of troponin and tropomyosin that is located on the thin (actin-containing) filaments (*1*). In contrast, in vertebrate smooth muscle, regulation is associated not with the thin actin filament but with the thick myosin filament. Here, calcium acts on calmodulin (CaM) which forms an active complex with myosin light chain kinase (MLCK)[1] and activates the contractile system via phosphorylation of the myosin regulatory light chain (*2*). Most likely, the latter system evolved from molluscan smooth muscles in which a direct binding of calcium by the myosin is required for the activation of contraction (*3*), a process in which myosin regulatory light chain (ReLC) also plays a major role (*4*). In addition to contraction, molluscan smooth muscles exhibit a unique mechanical state called the "catch state", characterized by an ability of the muscle to develop a long-lasting high-tension state with little energy expenditure (*5*, *6*). It is apparent that the ability to develop the catch state depends on the presence of major contractile proteins that are unique for these muscles. In addition to filamin, myosin, actin, and tropomyosin, the bivalve catch muscle contains large amounts of twitchin, paramyosin, or myorod (*7*). So far, the function of myorod has not been elucidated, although it is present in amounts equal to that of myosin. This heat stable protein is localized on the surface of thick filaments (*7*) together with myosin (*8*) and twitchin (*9*), and it interacts in vitro with the major components of thick filaments, namely, paramyosin, myosin, and twitchin (*10*, *11*).

Myorod is an alternative product of the myosin heavy chain (MHC) gene. It contains a rod part that is nearly identical to the rod portion of myosin and a unique N-terminal domain that bears some analogy to the regulatory light chain of myosin (*12*, *13*). Proteolytic digestion of myorod at low ionic strengths has confirmed that its structure is similar to that of myosin (*14*, *15*); i.e., there is a small head domain instead of a myosin head and a rodlike domain (analogous to the myosin rod). Digestion at high ionic strengths further splits the rod into a neck domain (analogous to the S2 fragment of myosin) and a tail domain identical to light meromyosin. The head domain of myorod (at the N-terminus) is much smaller than the myosin heads.

Like myosin, purified myorod assembles into filamentous aggregates, but the dependence of this aggregation on ionic

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[1]Abbreviations: MLCK, myosin light chain kinase; MA kinase, myosin-associated kinase; PKA, cAMP-dependent protein kinase A; TW−MY−MR complex, complex of proteins from molluscan smooth muscle composed of myosin, myorod, and twitchin; TW−MR complex, complex of twitchin and myorod; MHC, myosin heavy chain; ReLC, regulatory light chain of myosin.

Published on Web 04/19/2010

conditions and the properties of myorod filaments differ greatly from those of myosin (*10*, *16*), despite the identity of the C-terminal domains responsible for filament formation (*12*). An analogue of myorod, myosin rod protein (MRP), has been discovered in *Drosophila* muscles where it appears to play a structural role in myofibrillogenesis (*17*, *18*). Similar to myorod, MRP is an alternative product of the MHC gene. However, these proteins differ in the amino acid sequences of their unique N-terminal domains, and even the rod domain of the MPR is much larger.

Myorod has been found in all molluscan catch muscles investigated so far. Therefore, this protein represents an integral part of smooth bivalve muscles and may be related to the functional properties of these muscles (*7*), including the catch phenomenon (*10*, *12*). The hypothesis that myorod is involved in the catch phenomenon was challenged by the finding that myorod was not required for forming a catchlike state in an in vitro reconstitution system (*11*, *19*). In addition, it is unclear if the reconstitution was entirely successful because the system was not tested for the most important characteristics of the catch state, namely ATPase activities.

Originally, the catch phenomenon has been explained by the formation of cross-links between filaments within the contractile apparatus of molluscan smooth muscle (*20*). According to the "traditional model" (*21*), catch is related to the persistence of a calcium-free myosin cross-bridge strongly bound to actin filaments. An alternative "twitchin bridges hypothesis" suggests that twitchin provides an in vivo mechanical link between the thick and thin filaments independent of the myosin cross-bridges (*22*). The fundamental observations supporting this hypothesis are that phosphorylation of twitchin by a PKA-like kinase is required for termination of the catch state and that its dephosphorylation by a Ca/calmodulin-dependent phosphatase (calcineurin) is accompanied by formation of this state.

In this report, we provide data related to the existence of restrictive bindings between twitchin (located on F-actin) and myorod and myosin (located on the paramyosin core) that resulted in the formation of stable complexes between these major contractile proteins. Formation of these links was influenced by a protein kinase (first described in this study) endogenous to myosin. This novel kinase phosphorylated myosin only in the absence of Ca^{2+}, while the phosphorylation of myorod was independent of calcium, indicating the presence of a novel regulatory pathway, in addition to the one that phosphorylates twitchin.

EXPERIMENTAL PROCEDURES

Proteins. All contractile proteins were extracted from posterior adductor muscle myofibrils of the mussel *Mytilus edulis* prepared as described previously (*13*, *23*). Briefly, the dissected tissue was first homogenized in "bis wash" (BW) buffer: 40 mM KCl, 2 mM $MgCl_2$, 1 mM cysteine, 10 mM bis-Tris, and 10 mM imidazole-HCl (pH adjusted to 6.6) at 4 °C. After centrifugation (20 min, 10000 rpm in a GSA Sorvall rotor), the residue was resuspended and pelleted. This washing procedure was repeated twice. The pellet thus obtained represented a myofibril-like preparation that was extracted with 5 mM MgATP, 2 mM EGTA, and 0.5 M NaCl added to "AA buffer" [60 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 20 mM imidazole-HCl (pH adjusted to 7.0) at 25 °C], and the insoluble residue was removed by centrifugation as described for the washing. Such a freshly extracted actomyosin was then fractionated by addition of solid ammonium sulfate to yield myosin, twitchin, myorod, and their complex containing the endogenous kinase. The thin filament fraction (Figure 1b) obtained at 35% saturation was used as a source of myorod (*13*). The twitchin−myosin−myorod (TW−MY−MR) complex (Figure 1c) was purified from the 35−45% (Figure 1c) or 32.5−42.5% (Figure 1d) fraction after its solubilization and dialysis against the AA buffer to remove soluble proteins and resuspension of the insoluble pellet. Myosin was precipitated between 45 and 55% saturation, and it was purified by slow dialysis to form long filaments (Figure 1e). In a similar way, another TW−MY−MR complex was obtained that included the entire extracted myosin by broadening of the range between 35 and 60%. Myosin was removed from these complexes by a slow thawing and freezing cycle that resulted in myosin denaturation. Practically, the complex suspension (frozen in liquid nitrogen and stored at −70 °C) was placed inside a tightly closed polystyrene box together with six large cooling blocks also at −70 °C. The tightly closed box was kept at room temperature for 24 h (with the temperature slowly increasing to 1 °C), and then it was placed again in the −70 °C freezer overnight. After defrosting, the TW−MY−MR complex suspension turned into an insoluble pellet and was rinsed by resuspension in AA buffer and centrifuged for 15 min at 25000*g*. The twitchin and myorod (TW−MR) complex (Figure 1k) and the associated protein kinase (Figure 1l) were then extracted from the insoluble pellet with the AA buffer containing additional 0.5 M KCl and 2 mM EGTA, with removal of the denatured (insoluble) myosin by centrifugation (Figure 1i). After dialysis, the TW−MR complex together with the associated kinase was precipitated and collected by centrifugation. For a couple of preparations, the extraction and actomyosin fractionation was conducted at low ionic strengths according to the procedure developed by Sobieszek and Bremel (*23*). Except for the somewhat lower yield, the extracts (Figures 1m,n) and their fractions were essentially the same as those obtained at high salt concentrations.

Myorod's 11 kDa N-terminal peptide used in some experiments was obtained by mild chymotrypsin digestion (30 min at 25 °C) of the myorod suspension at an enzyme to myorod ratio of

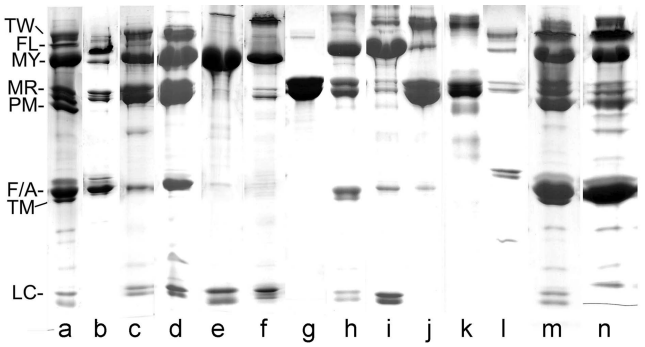

FIGURE 1: Contractile proteins and composition of supramolecular complexes used in this study: (a) posterior adductor actomyosin, (b) thin filament fraction, (c and d) TW−MY−MR complexes obtained at 35−45 and 32.5−42.5% saturation, respectively, (e) myosin (MY), (f) TW−MY complex fraction, (g) purified myorod (MR), (h) twitchin−myosin−myorod complex, (i) denatured myosin removed from the TW−MY−MR complex by freezing, (j and k) crude and final twitchin−myorod complex (TW−MR), respectively, (l) semi-purified MA kinase, and (m and n) initial actomyosins extracted from myofibrils at high and low ionic strengths, respectively (see Discussion).

1/1000 (*13*). After removal of undigested myorod by centrifugation, the peptide was purified by precipitation with acetone fractionation between 20 and 80% (v/v). The peptides such as protein kinase A inhibitor peptide PKI(5−24), cyclosporine A, and reagents (cAMP and ATP) were purchased from Sigma-Aldrich. The chemicals used were of the analytical or biochemical grade.

Gel-Filtration and Ion Exchange Chromatography. Purified filamentous myosin (Figure 1e) and the TW−MR complex (Figure 1c,d) stored at −70 °C were dissolved in AA buffer containing additional 0.5 M NaCl and 0.5 mM EGTA. After clarification by high-speed centrifugation, myosin (10 mg/mL), or the TW−MR complex (6 mg/mL) was loaded into a Sephacryl S-400 column (0.6 cm × 100 cm), equilibrated with the same buffer. Eluted fractions (1.8 mL) were dialyzed and analyzed for protein kinase activity using purified kinase-free myosin (5 mg/mL) or myorod (3 mg/mL) as a substrate in the presence of EGTA or calcium. The kinase-free myosin was obtained after precipitation of the first peak, while the combined kinase peaks from a few runs were subjected to ionic exchange chromatography on a small (0.6 cm × 5 cm) Q-Sepharose FF column, yielding the semipurified kinase.

Phosphorylation Assays. Endogenous kinase activities of mussel myosin (in the presence or absence of myorod) as well as those of the TW−MY−MR or twitchin−myorod complexes were measured in AA buffer containing additionally 0.06 M NaCl and 1 mM EGTA or 0.1 mM $CaCl_2$. Unless otherwise specified, PKA inhibitor PKI(5−24) was also added at a final concentration of 0.25 μM. The phosphorylation reaction (at 25 °C) was initiated by addition of 0.5−1 mM [γ-^{32}P]ATP to the assay mix and quenched with 8.5 M urea and 40 mM 4-(hydroxymercuril)benzoic acid. Full details of ^{32}P incorporation measurements and autoradiography as well as other procedures used are given in ref *13*.

Mass Spectrometric Analysis. Myorod and myosin used for the analysis were phosphorylated by semipurified kinase and then purified by gel-filtration chromatography on Sephacryl S-400 at a high ionic strength. After dialysis to remove the salt, the precipitated proteins were collected by centrifugation and resuspended in water. The phosphorylated or unphosphorylated proteins were enzymatically cleaved by endoproteinase Lys-C [EC 3.4.21.50] (Sigma-Aldrich, 1/20, w/w) in 100 mM NH_4HCO_3 buffer (pH 8.0) for 2 h at 37 °C. The digest was analyzed using nano-HPLC consisting of an UltiMate 3000 System (Dionex Corp.) connected online to a linear ion trap mass spectrometer (ThermoElectron Finnigan LTQ) equipped with a nanospray ionization source. The UltiMate system selectively enriched phosphopeptides. The nanospray voltage was set to 1.6 kV, and the heated capillary was held at 200 °C. Datum-dependent neutral loss MS^3 was used for the precise localization of phosphorylation. MS/MS and MS^3 spectra were searched against a chicken protein database using SEQUEST (LCQ BioWorks, ThermoFinnigan) and validated manually. The identified peptides were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides were as follows: Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charged ions, and Xcorr > 2.5 for triply charged ions. Only best matches were considered. MS/MS tolerances of ±0.6 Da were allowed. IMAC was performed using the SigmaPrep Spin Column kit and the PHOS-Select Iron Affinity Gel from Sigma.

RESULTS

Interaction among the Major Contractile Proteins. The protein composition of the posterior adductor muscle of *M. edulis* is different from that of many other muscle systems because its contractile apparatus includes relatively large amounts of proteins such as paramyosin, myorod, and twitchin. We expected that formation of complexes between these proteins with the common contractile proteins such as myosin and actin might give us clues for understanding the molecular mechanism of the catch state. Therefore, we have analyzed the protein composition of the adductor muscle extracted under relaxed conditions (at low and high ionic strengths) and fractionated by ammonium sulfate as well as by calcium or magnesium precipitation. As shown in Figure 1a, actomyosin extracted from relaxed myofibrils and precipitated by calcium and/or dialysis formed a complex that included twitchin, filamin, myosin, myorod, F-actin, and tropomyosin. These complexes may correspond to the catch or rigor state.

Ammonium sulfate fractionation of the freshly extracted actomyosin (with MgATP replenishment just before the fractionation) resulted in precipitation of the complexes of somewhat variable relative composition that depended on the saturation range. After more than 12 such extractions, fractionations, and purifications (see Experimental Procedures) with subsequent SDS−PAGE analysis, we established that the lower-saturation range pellet (0−35%) included a complex of filamin, myorod, and F-actin together with some twitchin and traces of myosin, the latter trapped in the 35% ammonium sulfate pellet (Figure 1b). It was surprising that such a thin filament fraction also contained a relatively high content of myorod (Figure 1b), a protein known to be associated with the thick filaments (*7*, *9*). This fraction represented a convenient initial source of myorod, the purification of which included removal of the proteins by denaturation at 80 °C, leaving the heat stable myorod in the supernatant (Figure 1g). In sedimentation experiments, we demonstrated that the coprecipitation of myorod with the thin filaments did not represent a true binding of myorod to F-actin but resulted from a coincidental precipitation of myorod at 35% saturation. Purified myorod was soluble at 5% but precipitated above 15% saturation. Similar binding behavior was exhibited by tropomyosin that was removed from F-actin at a low saturation level and coprecipitated with myosin at a high saturation level (see below).

The middle range complex (35−45%), purified by dialysis and precipitation, was composed of twitchin, myosin, and myorod as well as some F-actin (Figure 1c). A complex of the same composition but with a higher relative myorod content was obtained at a slightly lower saturation range between 32.5 and 42.5% (Figure 1d). Myosin precipitated together with tropomyosin over a higher saturation range (42.5−60 or 45−60%). The latter was readily separated from myosin after simple solubilization of the pellet and formation of myosin filaments by dialysis and their precipitation (Figure 1e). By modification of the saturation ranges, additional complexes composed of, for example, myosin, twitchin, and myorod at 35−55% were formed (Figure 1f). From the composition of these various complexes, it was apparent that under relaxed conditions, myorod was bound to myosin, and unexpectedly, twitchin appeared to be bound to myosin as well as to F-actin. All these complexes contained, in addition to the contractile proteins, variable levels of endogenous protein kinase as well as some of PKA-like kinase. The former represents the main subject of this study.

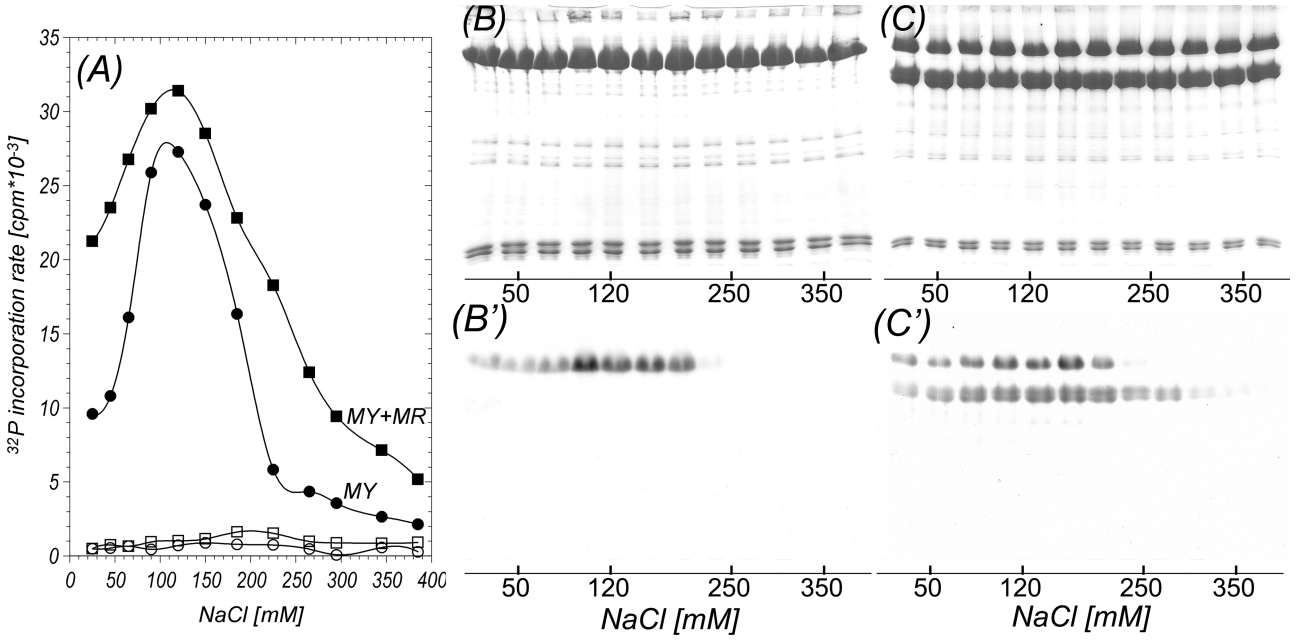

FIGURE 2: (A) Rates of phosphorylation of myosin and myorod by myosin MA kinase as a function of ionic strength in the absence (filled symbols) and presence (empty symbols) of calcium for purified myosin alone (circles) or with added purified myorod (squares). In this and the following analogous experiments, the ^{32}P incorporations were monitored in parallel by SDS−PAGE (B and C) and autoradiography (B′ and C′). Note that MA kinase phosphorylated myosin heavy chains (B′) and myorod (C′) but not the myosin light chain. The myosin concentration was 7.5 mg/mL and the myorod concentration 5.1 mg/mL.

Phosphorylation of Myorod and Myosin Heavy Chains by Myosin-Associated Kinase. Myosin obtained from ammonium sulfate fractionation and purified by dialysis and filament assembly exhibited protein kinase activity indicating the presence of a tightly bound myosin-associated kinase (MA kinase). This kinase phosphorylated both myosin and myorod (Figure 2), and it could not be removed by repetitive resuspension and pelleting of these filaments. In both cases, a very distinct optimum phosphorylation rate was observed around 120 mM NaCl, and an increase or decrease in the ionic strength resulted in a sharp reduction in these rates. SDS−PAGE and corresponding autoradiography demonstrated that MA kinase phosphorylated myorod and myosin heavy chains (MHCs), but not the regulatory light chains (ReLCs) of myosin (Figure 2B′,C′). This was not a molluscan form of protein kinase A (PKA) because its activity was unaffected by addition of 1−4 μM cAMP or by 0.05−10 μM specific PKA inhibitor PKI(5−24) (not shown). Therefore, it is apparent that the MA kinase was a novel kinase that may be involved in the Ca^{2+}-dependent regulation of the catch muscle.

Significantly, the activity of MA kinase was inhibited by calcium ions [Figure 3 (●)]. This inhibition could result, in principle, from activation of a Ca^{2+}-stimulated phosphatase, which dephosphorylated the proteins previously phosphorylated by the MA kinase. In fact, molluscan muscle cells contain a Ca^{2+}-stimulated phosphatase similar to vertebrate calcineurin (*24*, *25*) that is inhibited by cyclosporine A (*25*). Cyclosporine A was not able to change the inhibitory effect of Ca^{2+} on the activity of MA kinase in our system even at concentrations of 5−10 μM. Therefore, the inhibitory effect of Ca^{2+} must have a different basis.

At high salt concentrations and with Ca^{2+} removed by EGTA, the MA kinase was separated from the myosin by gel-filtration chromatography on Sephacryl S-400 with two peaks, the first one corresponding to myosin (protein peak) and the second representing the MA kinase activity peak (Figure 4). The activity peaks, measured with myorod or myosin as the substrate, were concurrent with each other [Figure 4 (□ and ○)], indicating that both proteins were phosphorylated by the same kinase. However, the amplitudes were different, and the one for myorod was ~2-fold higher than that for myosin. This indicates that myorod exhibited a 2-fold higher level of phosphorylation or contained additional sites in comparison to myosin. Most likely, the common sites were localized within the same C-terminal tip while the additional sites could also be found in the unique N-terminal domain of myorod (see below).

Consistent with this interpretation was the observed calcium dependence of the phosphorylation rates of myosin with added myorod [Figure 3 (● and ○, respectively)]. At a calcium concentration of < 0.1 μM, the rates with added myorod were approximately 2-fold higher than that of myosin alone, while there was a total inhibition of the phosphorylation at calcium concentrations of > 0.5 μM. Thus, calcium bound to myosin inhibited not only phosphorylation of myosin but also that of the myorod.

As expected, Ca^{2+} inhibition was also observed for the kinase fractions obtained during myosin chromatography on the S-400 column when myosin was used as the substrate, but unexpectedly, with the myorod there was no inhibition. As a result, the activity peaks obtained in the presence and absence of calcium were clearly different in their amplitudes and positions. At the lower descending shoulder of the myorod peak [Figure 4 (■)], the inhibition by Ca^{2+} was gradually removed, resulting in a very asymmetric shape of the peak. This clearly indicated that phosphorylation by the kinase was inhibited by myosin, and this inhibition was gradually removed in parallel with the disappearance of myosin traces in the fractions [Figure 4 (solid line)]. Thus, calcium was not inhibiting phosphorylation of myorod but only that of myosin. We investigated this question further by comparing the phosphorylation of myorod and myosin by semipurified MA kinase (Figure 5). In the presence of Ca^{2+} and PKA

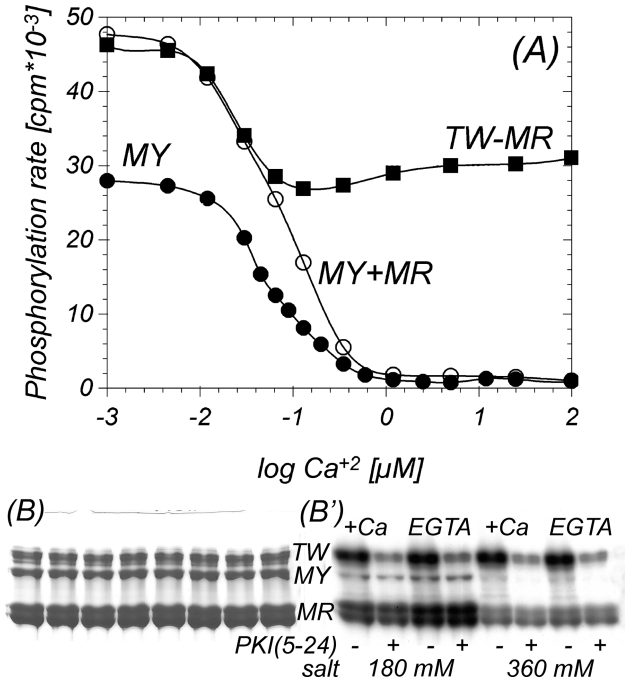

FIGURE 3: (A) Dependence of the phosphorylation rate on calcium concentration for purified myosin (●) and myosin with added purified myorod (○) as well as that of the TW−MR complex (■). Note that the activity of the MA kinase was inhibited at >0.1 μM Ca^{2+} with or without added myorod, while the rate of phosphorylation of the TW−MR complex was only partly inhibited. (B) SDS−PAGE and (B′) corresponding ^{32}P distributions at optimal (AA buffer with 120 mM NaCl) and high (AA buffer with 300 mM NaCl) salt concentrations. Note that addition of specific PKA inhibitor [PKI(5−24) peptide at 0.25 μM] inhibited phosphorylation of twitchin, but not that of myorod, at the optimal and high ionic strengths. The ^{32}P incorporations in the presence (0.1 mM) and absence (2 mM EGTA) are also shown. The calcium concentration was buffered with 5 mM Ca/EGTA buffer system with an apparent dissociation constant of EGTA for Ca^{2+} of 8.318×10^{-7} M. The concentrations were: myosin, 7.5 mg/mL; myorod, 4.0 mg/mL and TW-MR complex, 5 mg/mL.

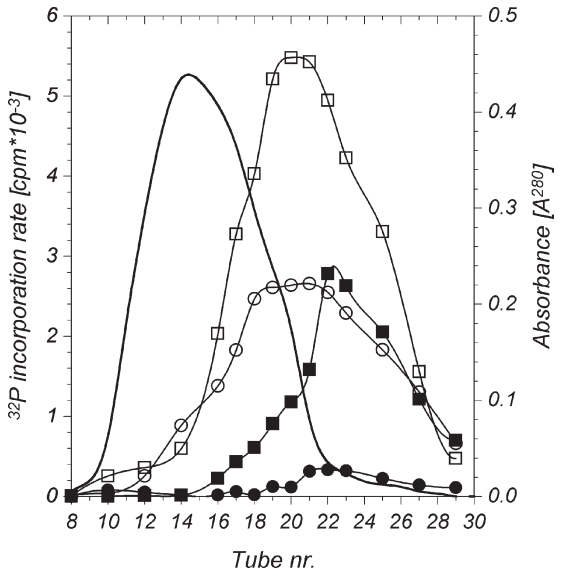

FIGURE 4: Partial separation of MA kinases by gel-filtration chromatography on a Sephacryl S-400 column at high ionic strengths. Kinase activities of the fractions were measured in the presence (filled symbols) or absence (empty symbols) of calcium using purified myosin (circles) or myorod (squares) as the substrate both at the final concentration of 2.5 mg/mL. The solid line corresponds to the absorbance at 280 nm. Note that MA kinase activities with myorod, in the absence (□) and presence (■) of calcium, did not comigrate, indicating inhibition of the phosphorylation by trace amounts of myosin present at the descending shoulder of the peak. Note also that in the absence of calcium there was a 2-fold higher level of incorporation for myorod (□) in comparison to that of myosin (○). For more details, see the text.

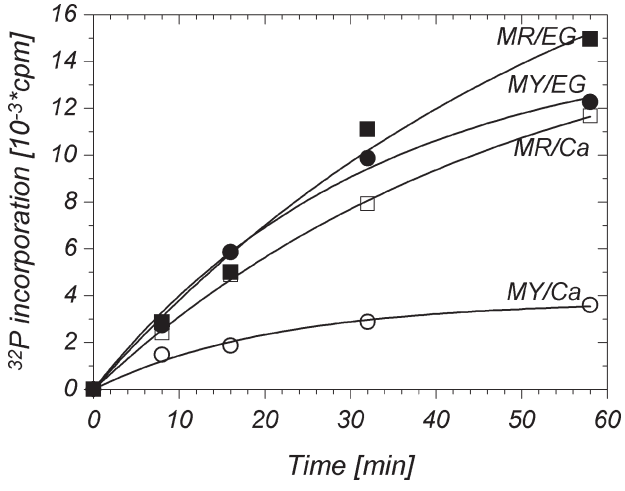

FIGURE 5: Phosphorylation of purified myosin and myorod by semipurified MA kinase in the absence and presence of calcium. Note a very low incorporation level of myosin in the presence of calcium (○) indicating that the MA kinase was not directly inhibited but that this inhibition resulted from binding of this cation by myosin.

inhibitor, phosphorylation of kinase-free myosin was inhibited, while when Ca^{2+} was removed by EGTA, the level of myorod phosphorylation was approximately equal to that of myosin (in Figure 5, compare filled circles with empty squares). Thus, as we concluded above, calcium acted directly on the substrate (myosin) and not on the enzyme (kinase). It would be more appropriate to conduct these experiments with the purified kinase, but this was not possible because, once separated from the myosin or myorod (see below), the MA kinase was very unstable, with a fast loss of its activity practically precluding its purification by ion exchange chromatography.

Kinase Activities of the Supramolecular Twitchin Complexes. Protein kinase was also present in the TW−MY−MR complex obtained from the 35−45% (or 32.5−42.5%) ammonium sulfate fraction (Figure 6). As in the case of purified myosin, this kinase could not be removed from the complex by simple washing at low salt concentrations. Similar to the data shown in Figure 2, the phosphorylation rates of this complex were inhibited at >240 mM NaCl (Figure 6A) but the optima were not as distinct as those for myosin (Figure 2A). This resulted from the somewhat stronger phosphorylation of twitchin and myorod above the 240 mM NaCl range that paralleled inhibition of the myosin phosphorylation (Figure 6B′,C′). Thus, it is apparent that the TW−MY−MR complex contained an additional protein kinase activity that phosphorylated twitchin and was not completely inhibited by 0.25 μM PKI(5−24) peptide which was always present in our assays. This was particularly clear at high ionic strengths (Figure 3B,B′) and may indicate that either the activity of this PKA-like kinase was not fully inhibited by the high salt concentrations (see below) or the phosphorylation resulted from twitchin autophosphorylation (*26*).

It has been previously reported that twitchin or its MLCK-like catalytic domain phosphorylates myosin ReLC (*13*, *27*). Therefore, twitchin could also contribute to the phosphorylation of

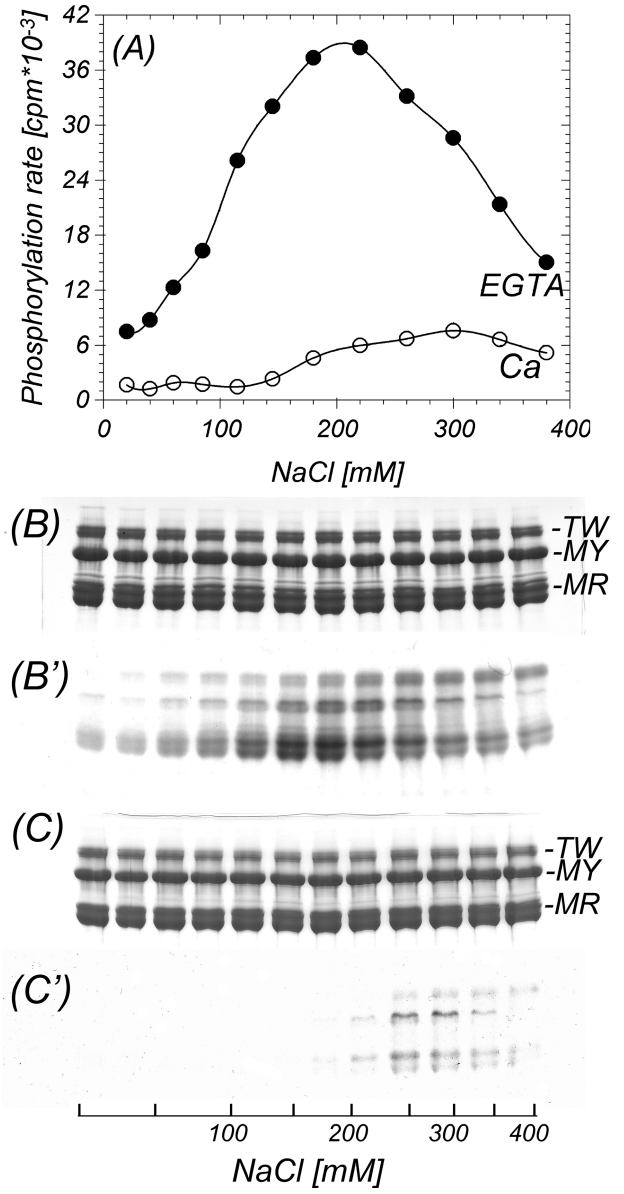

FIGURE 6: (A) Rates of phosphorylation of the TW−MY−MR complex as a function of ionic strength in the presence (○, 0.1 mM) and absence (●, 2 mM EGTA) of calcium. Note that at >240 mM NaCl, myosin heavy chains (MHC) were not phosphorylated. (B and C) SDS−PAGE and (B′ and C′) corresponding ^{32}P distributions. The TW−MY−MR complex concentration was 12 mg/mL.

myorod by phosphorylating its N-terminal, ReLC-like domain. To test this possibility, we purified the N-terminal 11 kDa peptide from myorod and added it to the TW complexes containing endogenous kinase [Figure 7 (filled symbols)]. Surprisingly, this peptide was not phosphorylated by the kinases associated with the TW complexes or by their twichin component [Figure 7 (empty symbols)], although, as expected, this peptide was readily phosphorylated by the Ca−MLCK complex [Figure 7 (◇)]. As shown in Figure 7 (○), the peptide inhibited phosphorylation of myorod and myosin within the TW−MY−MR complex. This demonstrated a strong interaction between the N-terminal domain of myorod and C-terminal tips of the myosin that may play a role in the catch state (see Discussion).

Inhibition of Phosphorylation within the Supramolecular Complexes. At high concentrations, the TW−MY−MR complex was extremely viscous, precluding any chromatographic separation of the components or their purification. In an alternative approach, most of the myosin was removed from this complex by a gentle denaturation via slow thawing and freezing (Figure 1h−j; see also Experimental Procedures). As a result, a different twitchin−myorod (TW−MR) complex was obtained in which myorod was phosphorylated at 5−10-fold higher rates. The incorporation levels were also much higher than these observed for the TW−MY−MR complex [Figure 3A (■)], approaching 1 mol of P_i/mol of myorod. Thus, the complex still contained the MA kinase, and it was obvious from the procedure that this kinase was now bound (coprecipitated) to myorod. From an approximately 2-fold loss of Ca^{2+} sensitivity of the inhibition, it was apparent that myorod was the major component that was phosphorylated. Ca^{2+} was not acting on the MA kinase but on the myosin, and myosin, in turn, also regulated phosphorylation of myorod. The TW−MR complexes also contained small amounts of myosin, phosphorylation of which was inhibited at high salt concentrations in contrast to that of myorod and twitchin (Figure 3B,B′). The phosphorylation pattern was complicated by a different degree of inhibition by salt. It was apparent that NaCl inhibited phosphorylation of myosin to the highest degree, followed by that of myorod and twitchin (Figure 3B,B′).

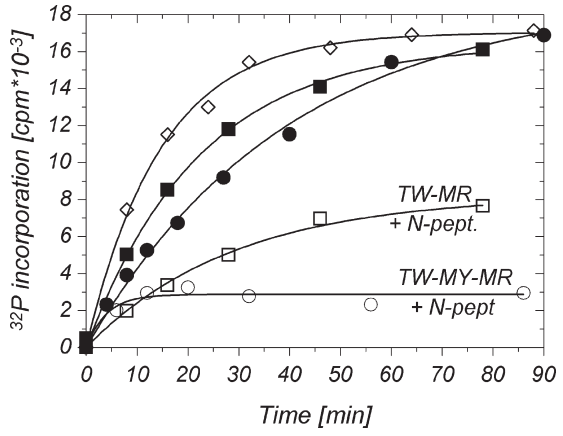

FIGURE 7: Phosphorylation progress of TW−MY−MR and TW−MR complexes in the presence and absence of the isolated N-terminal domain of myorod. Note that the relative level of incorporation for the TW−MR complex (■) was reduced ∼5-fold in the presence of the N-terminal peptide (□), while the reduction in the case of the TW−MR complex (●) was ∼2-fold (○). The isolated N-terminal tip of myorod was readily phosphorylated by purified gizzard MLCK (◇).

Reconstruction of the TW−MY−MR complex via addition of increasing amounts of kinase-free myosin to the TW−MR complex led to unexpected gradual inhibition of myorod phosphorylation even in the absence of Ca^{2+} (Figure 8B,B′). Upon addition, total phosphorylation rates in the absence of Ca^{2+} remained approximately constant, while those in its presence were inhibited. As clearly shown in the figure, the incorporation into the myosin compensated for inhibition (by myosin) of the incorporation into the myorod. In addition, myosin also inhibited phosphorylation of twitchin, and this was particularly clear in the absence of PKI(5−24) peptide when the PKA-like kinase activity was not inhibited (Figure 8B,B′, lanes 12−15). These are significant and novel observations demonstrating another regulatory effect of phosphorylated myosin on phosphorylation of myorod and twitchin. This also explains the relatively low levels of incorporation of the TW−MY−MR complexes observed in this study.

Identification of the Phosphorylation Sites. We concluded above that phosphorylation sites of MA kinase were localized

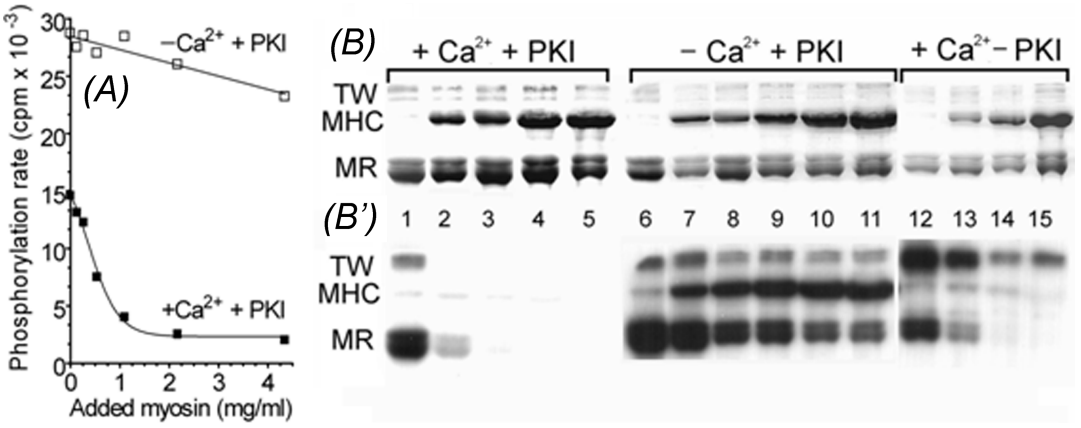

FIGURE 8: Inhibition of myorod and twitchin phosphorylation by filamentous myosin. (A) Dependence of the phosphorylation rate of the TW−MR complex on myosin concentration in the presence (■) or absence (□) of Ca^{2+}. Every second assay point from panel A was also analyzed by SDS−PAGE as shown in panel B, and the corresponding autoradiography is shown in panel B′. Note that concentration-dependent inhibition of myorod and twitchin phosphorylation by myosin was paralleled by an increased level of phosphorylation of myosin. Assays were conducted in AA buffer in the presence of 1 mM EGTA or 0.1 mM Ca^{2+}. Where indicated with a plus, 0.25 μM PKA inhibitor PKI(5−24) was added. The concentration of the TW−MR complex was 2 mg/mL.

within the common C-terminal tip of myosin and myorod. However, from the tight association of MA kinase with myosin filaments, and an analogous association of the vertebrate MLCK with myosin, we expected that the unique N-terminal (a light chain-like) domain of myorod could also be phosphorylated. To answer this question, we identified the myorod's sites phosphorylated by MA kinase using dynamic nanospray MS and MS/MS. Myorod was phosphorylated by semipurified protein kinase obtained from a soluble supernatant of the TW−MR complex after removal of denaturated myosin. We used this semipurified kinase preparation and not the one obtained from the kinase rich fractions of the Sephacryl S-400 column because the former was relatively stable while the activity of the latter was lost after separation from myosin during dialysis. After phosphorylation, myorod was purified on a Sephacryl S-400 column and then concentrated by precipitation, and digested with either endoproteinase LysC or endoproteinase GluC. Before the MS analysis, phosphorylated peptides from the digest were enriched using an immobilized metal affinity chromatography (IMAC) membrane. By this approach, two phosphorylation sites were identified in the unique N-terminal domain, Thr-102 (or Ser-103) and Thr-141 (or Ser-142), as well as two sites in the C-terminal tip of the molecule, Ser-970 (or Ser-971) and Ser-973. We showed previously that the Thr-141 site was phosphorylated by gizzard MLCK (*13*), while Ser-973 corresponds to the site identified by Castellani et al. (*28*) as being phosphorylated within the C-terminal tip of myosin. The same analysis was also conducted on unphosphorylated myorod which indicated that the Thr-102 (or Ser-103) and Thr-141 (or Thr-142) sites were already a partially phosphorylated state before the phosphorylation. Thus, MA kinase phosphorylated Ser-970 (or Ser-971) in addition to the previously shown phosphorylation of Ser-973 (*12*). Therefore, this C-terminal tip of myorod may play a significant role in its interaction with myosin (see Discussion).

We have also checked the phosphorylation of the N-terminal polypeptide by the same semipurified kinase. After SDS−PAGE, the isolated polypeptide exhibited three bands of ~18, ~15, and ~12 kDa with their total incorporation levels ~10-fold lower in comparison to these observed for the intact myorod. Analysis of the phosphorylated polypeptide together with autoradiography revealed that the three bands incorporated ^{32}P. These bands were cut from the gel, in-gel digested with trypsin, and analyzed by dynamic nanospray MS and MS/MS (*13*). All three bands included Thr-33 and Ser-65 in a partly phosphorylated state, in addition, the most intense band of the 15 kDa peptide showed that Thr-141 (or Ser-142) was also partly phosphorylated. Thus, it is clear that MA kinase or another kinase present in our semipurified preparation also phosphorylated the N-terminal domain of myorod, although to much lower levels.

DISCUSSION

Myorod, a molluscan catch muscle thick filament protein of unknown function, is an alternative product of the myosin heavy chain (MHC) gene. Its content in the catch muscles is nearly the same as that of myosin (*7*, *12*). We have shown previously that the unique N-terminal domain of this protein can be phosphorylated by gizzard MLCK (*13*). We initiated this study to examine whether a similar phosphorylation can also occur in vivo and to elucidate its regulatory function. The main result of our study is the identification and partial characterization of the novel kinase endogenous to molluscan catch muscle that phosphorylated myorod and myosin. The kinase was tightly bound to novel supramolecular complexes formed from twitchin, myosin, and myorod which appeared to be involved in the calcium regulation of molluscan smooth muscles. In addition, these complexes also included protein kinase A which is known to phosphorylate twitchin (*29*). Phosphorylation of twitchin inhibits and terminates the catch state (*5*). Thus, these two kinases, together with cAMP and Ca/calmodulin-dependent phosphatase (calcineurin-like), are responsible for the overall regulation of relaxation, active state, and catch.

The new kinase termed here myosin-associated kinase (MA kinase) phosphorylated myorod and MHC, but not regulatory light chains of myosin (Figure 2). Castellani and Cohen (*30*) observed a similar kinase in myosin preparations from molluscan adductor catch muscles, which also phosphorylated another unknown 110 kDa protein present in these myosin preparations. On the basis of the similarity in the amino acid sequences of chymotryptic peptides from this protein and those of myosin, the authors concluded that the unknown protein was the rod portion of myosin produced by proteolytic degradation during myosin

purification. It is now clear that this protein was myorod (*7*), also known as catchin (*12*), the main structure of which is identical to the C-terminal rod domain of myosin with an additional small N-terminal domain (*12*). This unique N-terminal domain bears some similarities to the myosin ReLC (*13*) (see also Figure 7). Consistent with these observations, we found that the MA kinase was also associated with myorod, but its native localization appeared to be myosin or the TW−MR complex; the latter is described for the first time in this study. At high salt concentrations, the kinase was separated from myosin and from the TW−MR complex, but it was denatured during myorod purification as a result of heat treatment. In agreement with the previous conclusions, we showed that MA kinase phosphorylated MHC and myorod in their nonhelical C-terminal tailpieces corresponding to Ser-971 and Ser-973 of myorod (*12*, *28*). Some phosphorylation also occurred at Thr-33, Ser-65, and Thr-141 (or Ser-142) when the isolated N-terminal domain was added to our semipurified MA kinase. However, we cannot exclude the possibility that these sites were phosphorylated by a different kinase because the MA kinase preparation used was not adequately purified.

Besides the tight association with myosin filaments, another important novel property of the MA kinase was inhibition of its activity by calcium ions as well as a distinct optimum of this activity at the physiological ionic strength (Figure 2A). The kinase was inhibited at >0.1 μM Ca^{2+} (Figure 3A), the concentration at which the muscle system became activated. This is the opposite of the vertebrate smooth muscles that are activated at these Ca^{2+} concentrations by MLCK, the latter also tightly associated with the myosin filaments (*31*, *32*). Although both kinases act on the same substrate (myosin), there is a fundamental difference between the molluscan and vertebrate smooth muscles. In the former case, Ca^{2+} activates myosin directly (*3*), while in the latter case, this ion acts indirectly on CaM to form a CaM−MLCK complex that activates myosin by phosphorylation of the ReLC. Significantly, we showed that it was not the kinase that was directly inhibited by Ca^{2+} but the substrate myosin that was blocked by this cation. It is known that when calcium binds, myosin undergoes a conformational change necessary for its interaction with F-actin. We also demonstrated that molluscan myosin regulated phosphorylation of twitchin and myorod, because it inhibited their phosphorylation within the TW−MR complex, but had no effect on the phosphorylation of myorod alone. Therefore, only under relaxation in the absence of Ca^{2+} were both myorod and myosin phosphorylated by MA kinase, resulting in their solubilization and their dissociation. Thus, it is apparent that some communication between myosin and myorod was taking place, and this resulted in modification of phosphorylation among these proteins (see below).

There is some analogy to the inhibition of *Acanthamoeba* MHC kinase via Ca^{2+}/calmodulin (*33*) where communication between the heads of the *Acanthamoeba* myosin and its C-terminal tails has been demonstrated (*34*). Such a communication is most obvious within the myosin antiparralel dimer, formation of which has been demonstrated for vertebrate smooth muscle (*35*). In addition, it has been shown that the antiparallel form is a building unit during filament assembly (*36*). It is very likely that similar heterodimers were formed between myorod and myosin because the latter inhibited phosphorylation of myorod while the isolated N-terminal peptide inhibited phosphorylation of these two contractile proteins. Thus, it is apparent that the flexible myorod's N-terminal domain and the C-terminal tailpiece of

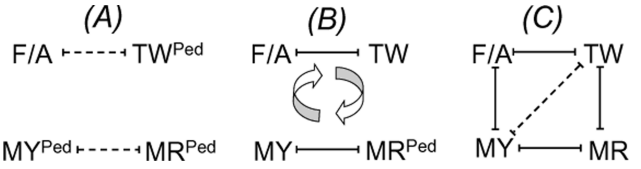

FIGURE 9: Scheme for interaction among the major contractile proteins in molluscan catch muscle in relaxed, active, and catch states. For more details, see Discussion.

myosin, together with their phosphorylation, may be responsible for the binding of these two proteins on the surface of the paramyosin core. Our demonstration of the existence of a TW−MY−MR complex and its phosphorylation confirmed earlier observations of the cross-links between the thin and thick filaments during the catch state and their abolishment after dephosphorylation of the twitchin (*11*). In the case of *Acanthamoeba* and *Dictyostelium*, phosphorylation of myosin not only decreases its actin-activated ATPase activity but also enhances solubility (*37*), and therefore, this is loosely associated with, or not bound to, the thick paramyosin filaments. This conclusion is supported by our observation that molluscan smooth muscle myosin can also be extracted under low-ionic strength conditions (Figure 1m,n), the same conditions that we use normally for extraction of vertebrate smooth muscle myosin (see ref *23*).

The phosphorylation sites of these myosins were shown to be located in the C-terminal tailpiece of the molecule that has been shown to favor myosin folding and increase its solubility (*38*). This is consistent with the in vitro experiments in which the phosphorylation level of myorod in synthetic thick filaments is lower under catch conditions (*24*). Accordingly, dephosphorylation of myosin may facilitate its assembly and increase the binding affinity for the paramyosin core required for development of the catch state. It has been concluded that twitchin, myosin, and myorod are surface proteins of the thick filament in catch molluscan muscles because twitchin interacts with myosin and myorod that, in turn, interact with each other (*22*) and with the paramyosin core (*9*, *11*). This is consistent with the formation of a stable TW−MY−MR complex demonstrated in this study and our observation that under relaxing conditions phosphorylation is taking place. The question arises whether the observed binding of myorod to twitchin (located on the thin filaments) is analogous to the binding of caldesmon to myosin in the vertebrate smooth muscle, providing a direct analogy between the catch state and tonic contraction. Indeed, there have been reports regarding the identification and phosphorylation of molluscan caldesmon (*39*), which most likely corresponds to myorod.

To summarize the possible implications of the presented data on the interaction between the major contractile proteins in the three states of molluscan catch muscles, we suggest that under relaxed conditions, MA and PKA kinases were active and therefore phosphorylated myosin, myorod, and twitchin result in a loose association of myorod with myosin and twitchin with F-actin (Figure 9 A). In the active state after the release of calcium, not only twitchin but also myosin becomes dephosphorylated by calcineurin because MA kinase was inhibited. Under these conditions, myorod is the only component that may stay phosphorylated since calcineurin does not act on this component (unpublished data; see also ref *19*). This enhances binding of myosin to myorod on the paramyosin core and that of twitchin to the thin filaments necessary for tension development

and/or shortening (Figure 9B). During the catch state with calcium removed, the demonstrated TW−MY−MR complex was cross-linked with the thin filaments (which were always bound to this complex), and the affinity of myorod for myosin further increased as a result of its dephosphorylation (Figure 9C). After the removal of calcium, slow phosphorylation of twitchin by PKA-like kinase and that of myosin and myorod by MA kinase may then result in relaxation (Figure 9A).

ACKNOWLEDGMENT

We thank Prof. Stefan Galler for his valuable suggestions that improved the clarity of the manuscript and Dr. Chun Y. Seow for the comments. We are grateful for a free donation of the *M. edulis* mussels by Vilsund Trading A/S in Denmark organized by Ms. Pia G. Jensen. A.S. thanks Prof. Beatrix Grubeck-Loebenstein for her constant support and encouragement as well as for her help in organizing the laboratory at the Life Science Center.

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