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Biochemistry

Effect of phosphorylation of smooth muscle myosin on actin activation and Ca^{2+} regulation

(kinase/phosphatase/myosin light chain/myosin ATPase/myosin-mediated regulation)

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ABSTRACT **A 35–70% ammonium sulfate fraction of smooth muscle actomyosin was prepared from guinea pig vas deferens. This fraction also contains a smooth muscle myosin kinase and a phosphatase that phosphorylates and dephosphorylates, respectively, the 20,000-dalton light chain of smooth muscle myosin. Phosphorylated and dephosphorylated smooth muscle myosin were purified from this ammonium sulfate fraction by gel filtration, which also separated the kinase and the phosphatase from the myosin. Purified phosphorylated and dephosphorylated myosin have identical stained patterns after sodium dodecyl sulfate/polyacrylamide gel electrophoresis. They also have similar ATPase activities measured in 0.5 M KCl in the presence of K^+-EDTA and Ca^{2+}. However, the actin-activated myosin ATPase activity is markedly increased after phosphorylation. Moreover, the actin-activated ATPase activity of phosphorylated myosin is inhibited by the removal of Ca^{2+} in the absence of any added regulatory proteins. Dephosphorylation of myosin results in a decrease in the actin-activated ATPase activity. Skeletal muscle tropomyosin markedly increased the actin-activated ATPase activity of phosphorylated but not dephosphorylated myosin in the presence, but not in the absence, of Ca^{2+}.**

Phosphorylation of a light chain of myosin has been shown to occur in myosins isolated from both muscle and cytoplasmic sources (1, 2). In each case, covalent phosphorylation occurs on a specific light chain of myosin in the size range of 18,000 to 20,000 daltons—e.g., white skeletal muscle, 18,000; red skeletal muscle, 20,000; cardiac muscle, 20,000; smooth muscle, 20,000; and platelet, 20,000 (1–3).

Phosphorylation is catalyzed by a specific enzyme, myosin light chain kinase, which differs in the cases of cytoplasmic and muscle myosin in that the former enzyme is independent of Ca^{2+} for activity whereas the latter enzyme requires Ca^{2+} (1, 4, 5). Dephosphorylation of the myosin light chain is catalyzed by an exogenous (6) or endogenous phosphatase (7, 28).

Phosphorylation of platelet myosin has been shown to control the interaction between platelet actin and myosin; phosphorylated myosin is activated by actin to a greater extent (5- to 7-fold) than is nonphosphorylated myosin (6). Dephosphorylation of platelet myosin results in a decrease in the actin-activated ATPase activity (6). Although these experiments indicated a role for phosphorylation in controlling actin-activation of myosin in nonmuscle cells, there was no evidence for such control in muscle.

Recent preliminary reports from a number of laboratories have suggested that phosphorylation of smooth muscle myosin may play a role in the actin-activation and Ca^{2+} regulation of smooth muscle myosin ATPase activity (8–10). In this paper we report the isolation and purification of phosphorylated and dephosphorylated forms of smooth muscle myosin from the guinea pig vas deferens. Myosin phosphorylation-dephosphorylation is shown to be under control of a kinase and a phosphatase that can be separated from myosin by gel filtration. Purified phosphorylated and nonphosphorylated myosins yield identical stained patterns after electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels. However, phosphorylated myosin has a much higher actin-activated ATPase activity than does nonphosphorylated myosin. Moreover, in the absence of troponin or other additional regulatory proteins, the actin-activation of phosphorylated myosin is dependent on the presence of Ca^{2+}.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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METHODS

All procedures were carried out at 4° unless noted as otherwise. Deionized water was used throughout.

Preparation of smooth muscle actomyosin

One-month-old male guinea pigs were anesthetized and their vasa deferentia were removed, collected in balanced salt solution (Hanks'), and carefully freed from connective tissue under a dissecting microscope. The cleaned specimens were pooled (usually 120 in number weighing approximately 5 g) and homogenized in 35 ml of the extraction buffer: 60 mM KCl, 40 mM imidazole·HCl (pH 7.1), 4 mM (ethylenedinitrilo)tetraacetic acid (EDTA), 10 mM ATP, and 10 mM dithiothreitol (DTT). A tissue homogenizer was used for a total of 2 min and the material being homogenized was chilled in ice. The homogenized material was sedimented at 48,000 × *g* for 20 min to yield a cloudy supernatant and a pellet. Immediately after addition of ATP and $MgCl_2$ to 10 mM, the supernatant was fractionated into 0–35% and 35–70% fractions by addition of a saturated ammonium sulfate solution in 10 mM EDTA (pH 7.0). The 35–70% ammonium sulfate fraction (10–15 mg/ml), which contained myosin, actin, and tropomyosin as well as other proteins, was used for phosphorylation, dephosphorylation, and purification of myosin. Prior to use, it was dialyzed overnight against 10–20 volumes of buffer containing 0.04 M KCl, 15 mM Tris·HCl (pH 7.5), and 5 mM DTT.

Phosphorylation and dephosphorylation of smooth muscle myosin

Phosphorylation and dephosphorylation were catalyzed by the endogenous kinase and phosphatase present along with the myosin in the 35–70% ammonium sulfate fraction. The incubation mixture contained 0.04 M KCl, 15 mM Tris·HCl (pH 7.0), 8 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM DTT, either 0.1 or 2.5 mM ATP, and [γ-^{32}P]ATP at 15–30 μCi/ml. Incubations were carried out at 23° for 1–3 min for phosphorylated samples and 20–30 min for dephosphorylated samples. Unphosphorylated myosin was prepared by incubating the 35–70% ammonium sulfate fraction in the absence of ATP.

Phosphorylation was terminated by raising the KCl concentration to 1 M, making the solution 0.1 M with respect to phosphate buffer, placing the samples on ice, and applying the samples to a Sepharose 4B column after 2 min of chilling.

Purification of myosin

Myosin was purified from the 35–70% ammonium sulfate fraction in both the phosphorylated and nonphosphorylated forms by gel filtration (6). A 1.5 × 90 cm column of Sepharose 4B equilibrated and eluted with 0.8 M KCl, 1 mM EDTA, 20 mM Tris·HCl (pH 7.5), and 5 mM DTT was used. Columns used for purification of phosphorylated myosin were equilibrated and eluted with 0.5 mM ATP added to the above buffer. Just prior to filtration, all samples were made 10 mM with respect to ATP and $MgCl_2$.

Biochemical assays

ATPase. Myosin ATPase activities were determined in 0.5 M KCl, 20 mM Tris·HCl (pH 7.5), 2 mM ATP, and either 2 mM EDTA or 10 mM $CaCl_2$, with a myosin concentration of 6 μg/ml. Actin-activated ATPase activity was measured in the presence of 10 mM imidazole·HCl (pH 7.2), 1 mM ATP, 1.6 mM $MgCl_2$, 20 mM KCl, and either 0.1 mM $CaCl_2$ or 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA). The following protein concentrations were used: myosin, 0.14 mg/ml; rabbit skeletal muscle actin, 0.5 mg/ml; rabbit skeletal muscle troponin-tropomyosin, 0.5–0.9 mg/ml; rabbit skeletal muscle tropomyosin, 0.5 mg/ml; rabbit skeletal muscle troponin, 0.5 mg/ml. Samples were incubated at 37°, and aliquots were removed at zero time and two additional times to ascertain linearity of phosphate release. Inorganic phosphate was measured by the method of Martin and Doty (11).

Kinase Assay. Kinase activity was assayed by using [γ-^{32}P]ATP and a platelet myosin light chain fraction as previously described (4).

Phosphatase Assay. Phosphatase was assayed as previously outlined (12). Samples (0.1–0.2 ml) were incubated with 2.0 mM *p*-nitrophenyl phosphate in 20 mM KCl, 4 mM $MgCl_2$, and 10 mM Tris·HCl (pH 7.3) at 37° for 1 hr; then, absorbance at 410 nm was measured. Phosphatase activity was also measured by incubating aliquots with ^{32}P-labeled platelet myosin light chains in the above buffer and monitoring ^{32}P-release with a Millipore filter assay (4).

$NaDodSO_4$/polyacrylamide gel electrophoresis

The incorporation, release, and exact location of ^{32}P was monitored by 1% $NaDodSO_4$/7.5% polyacrylamide gel electrophoresis according to Fairbanks *et al.* (13). Occasionally, methylene bis-acrylamide was replaced by *N,N'*-diallyltartardiamide (14). Following electrophoresis, gels were stained in 0.03% Coomassie brilliant blue. The stained gels were scanned at 584 nm with a Gilford model 2520 gel scanner and radioactivity was eluted as previously described (4, 14).

Preparation of other proteins

Rabbit skeletal muscle actin was prepared essentially as outlined by Spudich and Watt (15). Rabbit skeletal muscle troponin-tropomyosin was prepared by the method of Bailey (16) and was a gift of Dr. B. Barylko. Rabbit skeletal muscle tropomyosin and rabbit skeletal muscle troponin were prepared by the method of Eisenberg and Kielley (17) and were gifts of Drs. Yang and Eisenberg. The above proteins were assayed for biological activity by monitoring their effect on ATPase activity with rabbit skeletal muscle heavy meromyosin, a gift of Drs. Chock and Eisenberg, in the presence and absence of EGTA. They were also monitored for purity after $NaDodSO_4$/polyacrylamide gel electrophoresis. These proteins were assayed by the methods outlined above and shown to be free of kinase and phosphatase activities.

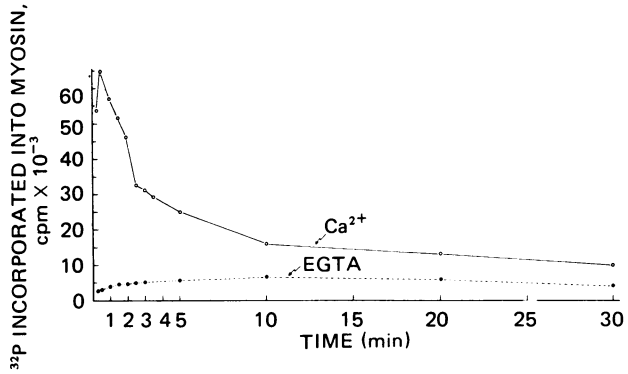

FIG. 1. Time course for the phosphorylation of vas deferens myosin. The 35–70% ammonium sulfate fraction was incubated in a total volume of 2 ml (final protein concentration, 2.5 mg/ml) in the presence of 0.1 mM ATP and either 0.1 mM $CaCl_2$ or 2 mM EGTA. Aliquots (50 μl) were removed at the indicated times and incorporation of ^{32}P was determined. Maximum incorporation in this preparation was 0.4 mol of P_i/mol of 20,000-dalton light chain.

Protein concentrations were determined by the procedure of Lowry *et al.* (18) with bovine serum albumin as a standard.

RESULTS

Preparation of phosphorylated and nonphosphorylated myosin

Three types of myosin were prepared: (*i*) unphosphorylated (i.e., myosin purified but not phsophorylated); (*ii*) phosphorylated; and (*iii*) dephosphorylated (myosin that was phosphorylated and then allowed to dephosphorylate in the presence of the endogenous phosphatase). Since unphosphorylated and dephosphorylated myosin gave similar results, they are sometimes grouped and referred to as "nonphosphorylated myosin."

Fig. 1 depicts a time course for the phosphorylation-dephosphorylation reaction. In the presence of 0.1 mM AT^{32}P there was an immediate rise and a rapid fall in the amount of ^{32}P incorporated into the 20,000-dalton light chain of myosin. Experiments using the Millipore filter assay to measure incorporation were monitored with 1% $NaDodSO_4$/7.5% polyacrylamide gel electrophoresis and showed that ^{32}P was incorporated only into the 20,000-dalton light chain. The rapid incorporation of ^{32}P followed by dephosphorylation suggested the presence of both kinase and phosphatase activities in the 35–70% ammonium sulfate fraction.

The kinase responsible for phosphorylating the 20,000-dalton light chain was dependent on the presence of Ca^{2+} for its activity (Fig. 1). This is in agreement with the findings of others (5, 8, 10).

To maximize the amount of phosphorylation of the 20,000-dalton light chain, the concentration of ATP in the phosphorylating mixture was increased to 2.5 mM. This allowed incorporation of approximately 0.6 mol of P_i/mol of light chain; it also resulted in a slower rate of dephosphorylation than that shown in Fig. 1.

Fig. 2 is an elution pattern obtained from gel filtration of a 35–70% ammonium sulfate fraction of phosphorylated myosin.

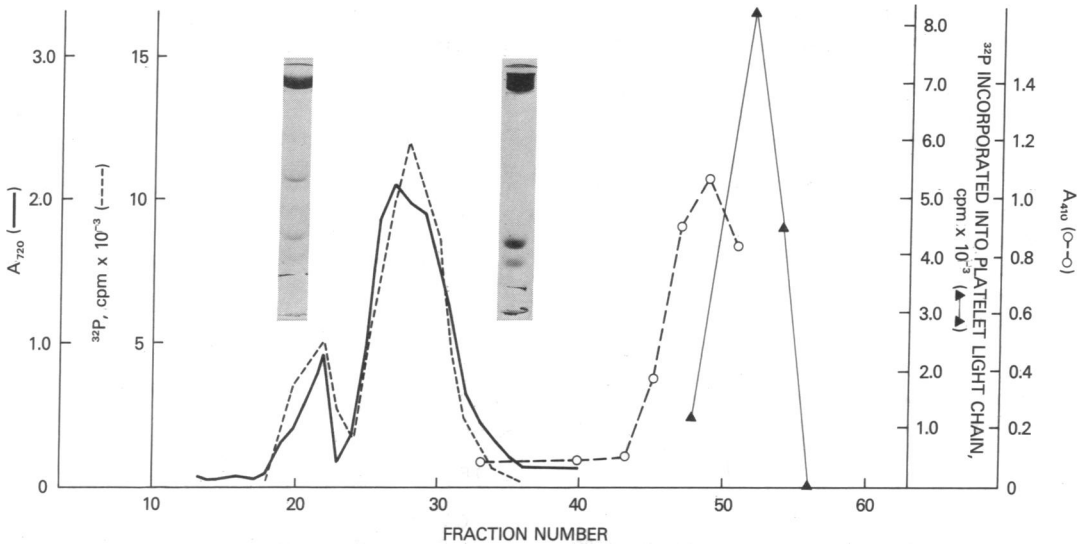

FIG. 2. Profile of Sepharose 4B gel filtration of the 35–70% ammonium sulfate fraction of smooth muscle actomyosin (18 mg of protein applied in 2 ml to a 1.5 × 90 cm column of Sepharose 4B). Fractions (2.5 ml) were collected at a flow rate of 13 ml/hr and were monitored for K^+-EDTA stimulated ATPase activity (A_{720}), ^{32}P radioactivity, kinase activity (^{32}P incorporated into platelet myosin light chain), and phosphatase activity (A_{410}). The stained 1% NaDodSO$_4$/7.5% polyacrylamide gels are of the first pooled peak of ATPase activity (fractions 19–23) at the left and the second peak (fractions 27–32) at the right. Migration of proteins is from top to bottom; stained bands from the second peak correspond to myosin heavy chain (200,000 daltons) and myosin light chains (20,000 and 16,000). Actomyosin is present in the first peak. Void volume = 45 ml; salt boundary = 155 ml.

The elution volumes for actomyosin (fractions 19–23), myosin (fractions 27–32), phosphatase (fractions 44–52), and kinase (fractions 48–54) are shown on the profile. The column-purified myosin samples, unlike the crude 35–70% ammonium sulfate fraction, were free of kinase and phosphatase activities. Lack of phosphatase activity in column-purified samples of myosin that had been previously phosphorylated was also indicated by the stabilization of covalently bound radioactivity as measured by elution from NaDodSO$_4$/polyacrylamide gels.

Figs. 3 and 4 show scans, at 584 nm, of NaDodSO$_4$/polyacrylamide gels as well as ^{32}P elution profiles from the gels for phosphorylated and dephosphorylated myosin. Fig. 3A is the gel profile of a 35–70% ammonium sulfate fraction that was phosphorylated for 2 min just prior to gel filtration. Fig. 3B is a scan of the myosin peak from the same sample after elution from agarose (see Fig. 2). The specific radioactivity of the phosphorylated light chain (LC-1) was found to be unchanged after agarose filtration.

Fig. 4 is a composite of gel scans and radioactivity profiles from a sample that was phosphorylated and then allowed to dephosphorylate prior to gel filtration. Fig. 4A is a scan of an aliquot removed from the phosphorylating mixture after only 2 min of incubation. The sample was allowed to dephosphorylate for 20 min and then was chromatographed on Sepharose 4B. Fig. 4B shows the dephosphorylated myosin after Sepharose 4B chromatography. Note that dephosphorylated myosin resembled phosphorylated myosin (Fig. 3B) in its stained profile but no longer contained a radioactive peak at LC-1.

Enzymatic properties of phosphorylated and nonphosphorylated myosin

Table 1 compares the ATPase activity of phosphorylated and nonphosphorylated myosins. At high ionic strength, there was no significant difference in the K^+-EDTA and Ca^{2+} activated ATPase activities between phosphorylated and nonphosphorylated myosins (last two columns, Table 1). In contrast, the actin-activated ATPase activities for the phosphorylated and nonphosphorylated samples were markedly different. Phosphorylation resulted in a marked increase in the actin-activated ATPase activity when assayed in the presence of Ca^{2+}.

Table 1 also demonstrates that purified phosphorylated myosin is dependent on Ca^{2+} for actin-activation because replacing Ca^{2+} by EGTA resulted in inhibition of the actin-activated ATPase activity. This activation was dependent on the presence of Ca^{2+} in the absence of any added regulatory proteins (see Figs. 3B and 4B). As outlined in the *Methods* section,

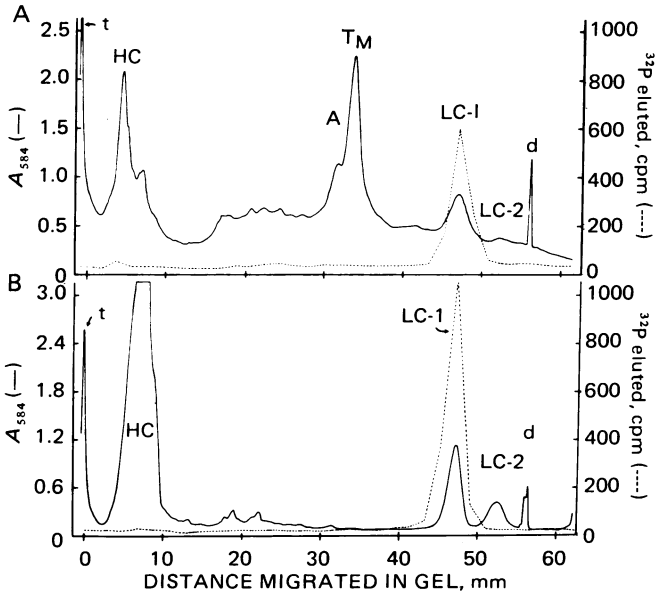

FIG. 3. Gel scans and radioactivity elution profiles of the phosphorylated 35–70% ammonium sulfate fraction (A) and purified phosphorylated myosin peak from Sepharose 4B chromatography (B). The 1% NaDodSO$_4$/7.5% polyacrylamide gels stained with Coomassie brilliant blue were scanned at 584 nm, and ^{32}P was eluted from 2-mm slices. Migration in the gels is from left to right. *t*, gel top; *HC*, myosin heavy chain; *A*, actin; *TM*, tropomyosin; *LC-1*, myosin light chain (20,000 daltons); *LC-2*, myosin light chain (16,000 daltons); *d*, dye marker.

Table 1. Effect of phosphorylation on myosin ATPase activity

Preparation	Myosin*	+Actin (0.02 M KCl) Ca^{2+}	+Actin (0.02 M KCl) EGTA	Myosin alone (0.5 M KCl) K^+-EDTA	Myosin alone (0.5 M KCl) Ca^{2+}
1	P	0.040	0.017	1.64	0.89
	U	0.012	0.008	1.45	0.81
2	P	0.085	0.028	1.50	0.87
	D	0.010	0.009	1.36	0.96
	D	0.007	0.005	1.10	0.84
3	P	0.105	0.034	1.45	0.68

The assay conditions are described in *Methods*. Activity is expressed in μmol P_i released/mg of myosin per min

* P = phosphorylated; U = unphosphorylated; D = dephosphorylated.

the rabbit skeletal muscle actin used in these studies was free of tropomyosin and troponin. It is important to note that, when ^{32}P-labeled myosin was used in these ATPase assays, the specific radioactivity of the labeled 20,000-dalton myosin light chain remained constant during the entire time course.

Addition of rabbit skeletal muscle troponin-tropomyosin, tropomyosin alone, or tropomyosin and troponin as separate proteins resulted in a significant increase in the actin-activated ATPase activity in the presence of Ca^{2+} (compare the actin-activated ATPase activities in Tables 1 and 2). Tropomyosin and troponin-tropomyosin had no such effect on nonphosphorylated myosin. Of particular interest is the finding that, on the addition of tropomyosin without troponin, the actin-activation of phosphorylated myosin was four times higher in the presence of Ca^{2+} than in the presence of EGTA.

DISCUSSION

Published values for the actin-activated ATPase activity of smooth muscle myosin have in general been quite low—e.g., 0.014 μmol of P_i/mg of protein per min for horse esophagus smooth muscle myosin (19) and 0.028 μmol of P_i/mg of protein per min for acto-heavy meromyosin from arterial smooth muscle (20).

The finding that phosphorylation of platelet myosin by an endogenous kinase resulted in a 5- to 7-fold increase in actin-activated myosin ATPase activity suggested that a similar mechanism might be involved in actin activation of smooth muscle myosin (6). Moreover, in a recent study, the purified

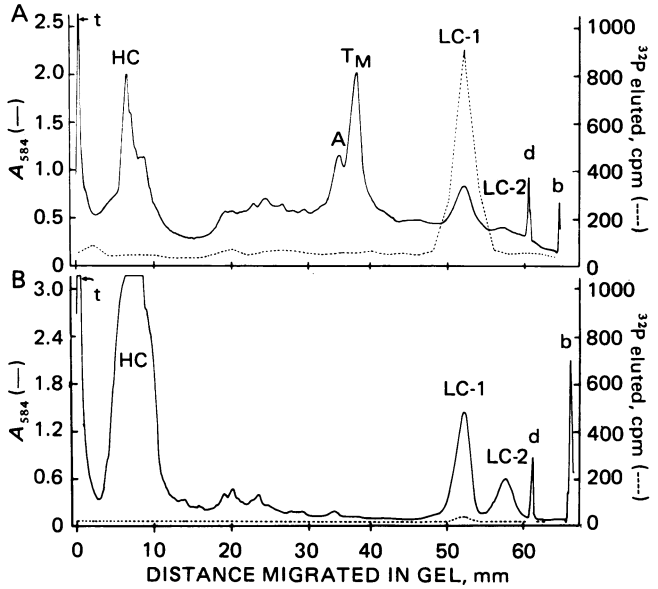

FIG. 4. Gel scans and radioactivity profiles of the 35–70% ammonium sulfate fraction (A) and purified myosin peak after dephosphorylation and subsequent Sepharose chromatography (B). (A) shows that the myosin was originally phosphorylated. (B), in contrast to Fig. 3B, shows a marked decrease in ^{32}P associated with LC-1, indicating that the myosin was dephosphorylated. Gel migration was from left to right. The abbreviations are as in Fig. 3, except *b*, gel bottom.

myosin light chain kinase from platelets was found to be capable of phosphorylating the 20,000-dalton light chain of chicken gizzard smooth muscle myosin (4).

Working with chicken gizzard myosin, Sobieszek (8) showed a relationship between actin activation of smooth muscle myosin and phosphorylation. Following his report, Aksoy *et al.* (21) and Górecka *et al.* (10) demonstrated that addition of a crude kinase from chicken gizzard to chicken gizzard actomyosin resulted in an increase in myosin phosphorylation and in actin-activated myosin ATPase activity. Neither report mentioned the effect of phosphorylation using purified smooth muscle myosin free of kinase and phosphatase.

In this paper we report on the properties of purified phosphorylated and dephosphorylated myosins isolated from the vas deferens of the guinea pig. The 35–70% ammonium sulfate fraction of a low ionic strength extraction was found to contain both a kinase and a phosphatase. The kinase, which was dependent on Ca^{2+} for its activity (Fig. 1), catalyzed the phos-

Table 2. Effect of troponin-tropomyosin and tropomyosin on phosphorylated and nonphosphorylated myosins

Preparation	Myosin*	+Actin +troponin-tropomyosin Ca^{2+}	+Actin +troponin-tropomyosin EGTA	+Actin +tropomyosin Ca^{2+}	+Actin +tropomyosin EGTA	+Actin +tropomyosin +troponin Ca^{2+}	+Actin +tropomyosin +troponin EGTA
1	P	0.135	0.026				
	U	0.012	0.010				
2	P	0.150	0.049	0.158	0.039	0.153	0.020
	D	0.025	0.009	0.014	0.010	0.015	0.010
	D	0.014	0.005				
3	P	0.183	0.013				

The assay conditions are described in *Methods*. Activity is expressed as μmol of P_i released/mg of myosin per min. The actin-activated ATPase activities in Table 1 should be compared with the respective preparation in Table 2.

* P = phosphorylated; U = unphosphorylated; D = dephosphorylated.

phorylation of the 20,000-dalton light chain of vas deferens myosin. Although the extent of phosphorylation was limited by the presence of a phosphatase, phosphorylation of 0.6 mol of light chain was achieved with this preparation [based on the assumption that, similar to skeletal muscle myosin, only a single site in the 20,000-dalton light chain is phosphorylated (22)].

Incubating the 35–70% ammonium sulfate fraction at 23° in the presence of ATP and $MgCl_2$ for 2 and 20 min permitted the isolation of a phosphorylated and a dephosphorylated form of myosin. These two forms of myosin could be purified, as well as stabilized with respect to phosphorylation, by gel filtration on Sepharose 4B because this step separates myosin from the kinase and phosphatase (Fig. 2). After gel filtration, the purified myosin peaks were found to be free of kinase and phosphatase activities.

Phosphorylation of smooth muscle myosin led to a marked increase in the actin-activated ATPase activity measured in 0.02 M KCl. Moreover, in an assay system utilizing rabbit skeletal muscle actin and purified phosphorylated smooth muscle myosin, this activation was dependent on Ca^{2+}. Thus, calcium regulation of the contractile cycle in smooth muscle, similar to molluscan muscles (23), appears to be mediated by myosin. This was originally suggested for gizzard actomyosin by Bremel (24) and later by Sobieszek and Small (25) on the basis of less direct evidence.

Recently, Górecka *et al.* suggested that the Ca^{2+}-sensitive regulatory mechanism of gizzard actomyosin is mediated via the kinase. Those studies were carried out by adding a crude kinase, made from gizzard native tropomyosin, to actomyosin. The properties of purified phosphorylated myosin were not examined. The work reported here demonstrates that Ca^{2+} regulation is an inherent property of the phosphorylated myosin molecule. The present work is also the initial demonstration that the marked effect of phosphorylation on actin activation of smooth muscle myosin is readily reversible by an endogenous phosphatase. Thus, the activation, relaxation, and Ca^{2+} regulation of myosin from vas deferens can be related to the phosphorylating-dephosphorylating system.

Because smooth muscle myosin contains two different light chains (20,000 and 16,000 daltons), it is possible that, although phosphorylation of the 20,000-dalton light chain results in actin activation of myosin, Ca^{2+} sensitivity could be mediated by either one or both light chains. The finding by Kendrick-Jones *et al.* (26) that the 20,000-dalton light chain of chicken gizzard myosin restores full calcium binding and also resensitizes purified scallop myosin strongly suggests that the phosphorylated light chain is responsible for Ca^{2+} sensitivity.

Although the values reported in Table 1 indicate a marked increase in actin activation for phosphorylated myosin compared to nonphosphorylated myosin, there is also a significant difference among the phosphorylated preparations themselves. The most likely cause of this difference is incomplete phosphorylation. The values for preparations 2 and 3 were obtained using 2.5 mM ATP (0.1 mM for preparation 1) in the incubation mixture and resulted in an incorporation of 0.5–0.6 mol of P_i per mol of myosin light chain.

Table 2 demonstrates that native tropomyosin (troponin-tropomyosin) increases the actin-activated ATPase activity of phosphorylated smooth muscle myosin. This effect, which is shown to be due to tropomyosin alone (Table 2, columns 3 and 4), is similar to the effect of tropomyosin on the actin activation of myosin reported for other contractile systems (27). Of particular note here is the finding that the stimulation was only observed when phosphorylated myosin was used. Similar to the findings with actin alone, the actin-activated ATPase activity in the presence of tropomyosin was inhibited by EGTA.

Phosphorylation of myosin has been demonstrated to play an important regulatory role in actin-myosin interaction in both smooth muscle and blood platelets. Two major differences between these contractile systems are: (*a*) the kinase that phosphorylates smooth muscle myosin is dependent on the presence of Ca^{2+} for activity whereas the platelet kinase is not (4), and (*b*) the Ca^{2+} regulation of actin activation of myosin ATPase activity is mediated through myosin in smooth muscle (as shown here) but is not mediated by myosin in platelets (28).

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