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Ca^{2+}- and Calmodulin-Dependent Stimulation of Smooth Muscle Actomyosin Mg^{2+}-ATPase by Fodrin†

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ABSTRACT: Fodrin, a spectrin-like actin and calmodulin binding protein, was purified to electrophoretic homogeneity from a membrane fraction of bovine brain. The effect of fodrin on smooth muscle actomyosin Mg^{2+}-ATPase activity was examined by using a system reconsituted from skeletal muscle actin and smooth muscle myosin and regulatory proteins. The simulation of actomyosin Mg^{2+}-ATPase by fodrin showed a biphasic dependence on fodrin concentration and on the time of actin and myosin preincubation at 30 °C. Maximal stimulation (50–70%) was obtained at 3 nM fodrin following 10 min of preincubation of actin and myosin. This stimulation was also dependent on the presence of tropomyosin. In the absence of myosin light chain kinase, the fodrin stimulation of Mg^{2+}-ATPase could not be demonstrated with normal actomyosin but could be demonstrated with acto–thiophosphorylated myosin, suggesting that fodrin stimulation depends on the phosphorylation of myosin. Fodrin stimulation was shown to require the presence of both Ca^{2+} and calmodulin when acto–thiophosphorylated myosin was used. These observations suggest a possible functional role of fodrin in the regulation of smooth muscle contraction and demonstrate an effect on Ca^{2+} and calmodulin on fodrin function.

Fodrin is a protein whose physical, biochemical, and immunological properties are similar to those of spectrin, one of the major cytoskeletal components found in erythrocytes (Goodman et al., 1981). It is composed of two nonidentical subunits (M_r 240000 and 235000) intertwined in a filamentous form in the cell. Purified fodrin appears to exist as a tetramer consisting of two heterodimers joined head to head under physiological conditions (Bennett et al., 1982). It binds actin filaments and undergoes Ca^{2+}-dependent association with calmodulin (Palfrey et al., 1982). Some investigators (Kakiuchi et al., 1982; Davies & Klee, 1981) purified fodrin from mammalian brain as a calmodulin binding protein without knowing its relationship to spectrin; therefore, it was also called calmodulin binding protein I or calspectin.

Fodrin is concentrated in regions underlying the plasma membrane of neuronal and a wide variety of nonneuronal cells and tissues [for a review, see Goodman & Zagon (1984)]. It

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exists in association with other cytoskeletal and contractile proteins to form a mobile membrane lining. In neuronal cells, fodrin moves along the axon at a rate similar to actin, myosin, and microtuble proteins (Levine & Willard, 1981). When certain cells such as fibroblasts and lymphocytes are challenged by multivalent ligands (surface immunoglobulin and concanavalin A) to induce membrane receptor capping, fodrin is shown to form patches (subcaps) along with actin, myosin, α-actinin, and possibly calmodulin (Levine & Willard, 1983; Nelson et al., 1983). The comigration of fodrin with contractile proteins suggests that this protein may be part of a nonmuscle contractile system, although no direct evidence supporting such a suggestion is available and the mechanism whereby fodrin may be involved in such a system is not known. It has been demonstrated by in vitro studies that fodrin can affect certain actin-dependent processes including polymerization of G-actin (Sobue et al., 1982), cross-linking of F-actin (Glenney et al., 1982), and the modulation of skeletal muscle actomyosin Mg^{2+}-ATPase activity (Shimo-Oka & Watanabe, 1981; Wagner, 1984). None of these effects has been shown to be regulated by calmodulin, and it is not clear whether any of these effects are related to the physiological action of fodrin.

Although smooth and nonmuscle myosins are distinct, they share some common structural and functional properties which are different from those of skeletal and cardiac muscle proteins (Adelstein & Esienbert, 1980). The regulation of smooth and nonmuscle actomyosin Mg^{2+}-ATPase activity by Ca^{2+} is believed to be mediated primarily by the calmodulin-dependent myosin light chain kinase which catalyzes the phosphorylation of a pair of myosin light chains of molecular weight 20000 (LC20)[1] (Dabrowska et al., 1978). When the cytosolic Ca^{2+} concentration of smooth muscle is increased, the velocity of shortening correlates with the phosphorylation state of LC20. Biochemical and physiological evidence suggests the existence of at least one secondary Ca^{2+}-dependent mechanism which can modulate actin–myosin interactions and thereby the contractile state of smooth muscle (Chacko & Rosenfeld, 1982; Nonomura & Ebashi, 1980; Nishikawa et al., 1983; Aksoy et al., 1983; Chatterjee & Murphy, 1983). In this study, we show that fodrin is capable of stimulating smooth muscle actomyosin Mg^{2+}-ATPase in a Ca^{2+}-and calmodulin-dependent manner. This is the first demonstration of a Ca^{2+}-and calmodulin-dependent fodrin effect. The stimulation of actomyosin Mg^{2+}-ATPase by fodrin also depends on other specific conditions, including a critical concentration of fodrin, preincubation of actin and myosin, an active state of myosin, and the presence of tropomyosin.

Experimental Procedures

[γ-^{32}P]ATP (10 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario). ATPγS was purchased from Boehringer Mannheim (Dorval, Quebec). Rabbit skeletal muscle actin and chicken gizzard myosin, tropomyosin, and myosin light chain kinase were purified following established procedures (Zot & Potter, 1981; Persechini & Hartshorne, 1981; Bretscher, 1984; Ngai et al., 1984). Myosin was stored on ice and not used beyond 3 weeks of preparation. The actin-activated Mg^{2+}-ATPase activity of gizzard myosin undergoes a slow loss of activity over this period, so the absolute ATPase rates obtained in experiments conducted at different times cannot be compared directly (P. K. Ngai and M. P. Walsh, submitted for publication). Calmodulin and calmodulin-dependent phosphodiesterase were purified as described previously (Sharma et al., 1979, 1980).

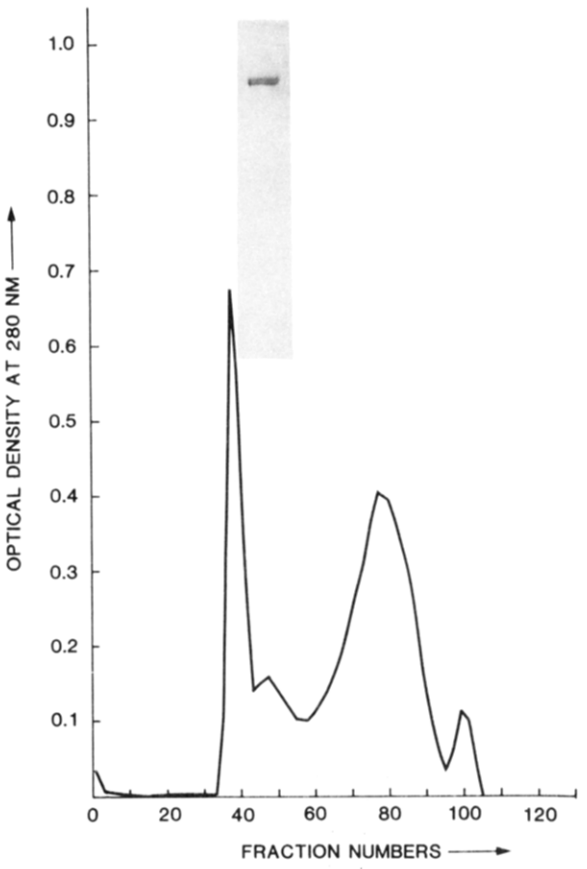

FIGURE 1: Fodrin purification: Sepharose 4B column chromatography. The final stage of fodrin purification involved gel filtration on Sepharose 4B (see Experimental Procedures). Protein elution was monitored by recording A_{280} (—). Fodrin-containing fractions were pooled (fractions 46–53), examined by 5–20% SDS–PAGE (see inset), and used for subsequent experiments.

Purification of Bovine Brain Fodrin. Bovine brain fodrin was purified by the method of Glenney et al. (1982) or with the following modifications. Frozen bovine brain was homogenized in buffer A (10 mM Tris-HCl, pH 7.5, 15 mM β-mercaptoethanol, 1 mM PMSF, and 5 mM EGTA), and the membrane fraction was obtained by centrifugation at 150000*g* for 1 h. Proteins were extracted from the membrane fraction in buffer B (20 mM Tris-HCl, pH 7.5, 15 mM β-mercaptoethanol, 1 mM PMSF, 5 mM EGTA, and 0.6 M KCl) and centrifuged at 150000*g* for 1 h. The supernatant was precipitated with ammonium sulfate to 30% saturation, and precipitated proteins were sedimented at 100000*g* for 30 min. To the supernatant, more ammonium sulfate was added to 45% saturation, and precipitated proteins were sedimented at 100000*g* for 30 min. The pellet was dissolved in a minimum volume of buffer B with the aid of a hand-operated Potter–Elvehjem homogenizer and dialyzed overnight against two changes (4 L each) of buffer B. The dialyzed sample was centrifuged at 100000*g* for 15 min to remove undissolved material. The supernatant was applied to a column (5 × 90 cm) of Sepharose 4B equilibrated with buffer B at a speed of 20 mL/h. The protein content in each fraction (2.5 mL) was determined by the absorbance at 280 nm, and selected fractions were examined by 0.1% SDS, 5–20% polyacrylamide

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, DL-dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MLCK, myosin light chain kinase; LC20, 20000-dalton subunit of myosin; TM, tropomyosin; CaM, calmodulin; A, actin; M, myosin; F, fodrin; Da, dalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

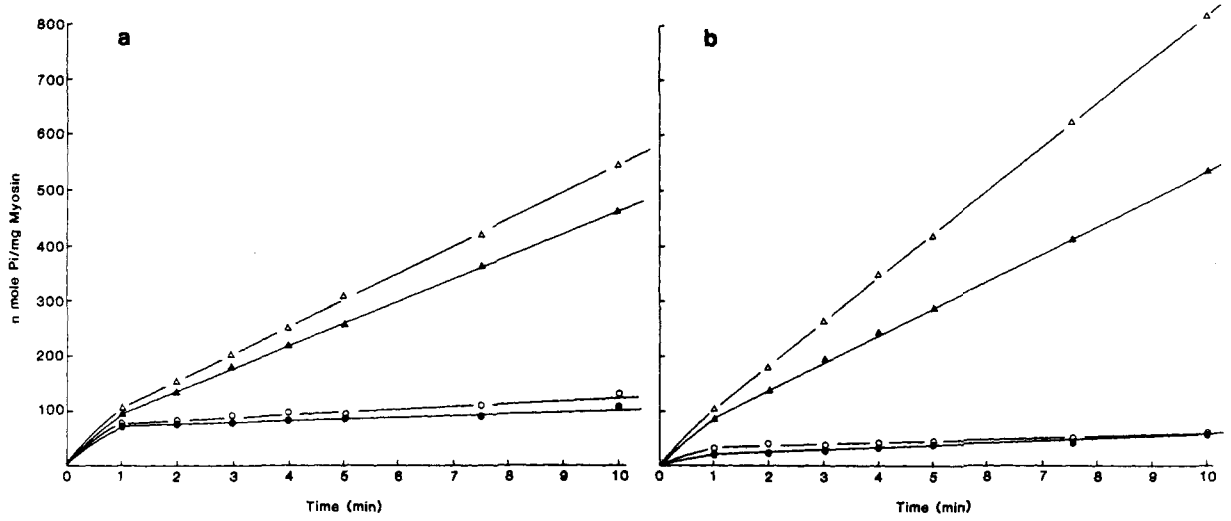

FIGURE 2: Stimulation of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity by fodrin. Actin (0.25 mg/mL), myosin (0.44 mg/mL), myosin light chain kinase (10 μg/mL), tropomyosin (50 μg/mL), and calmodulin (10 μg/mL) were preincubated with (△, ○) or without (▲, ●) fodrin (3 μg/mL) at 30 °C for 2 min (a) or 10 min (b) prior to the addition of [γ-^{32}P]ATP to initiate the ATPase reactions. ATP hydrolysis was measured as described under Experimental Procedures in the presence of 0.1 mM Ca^{2+} (△, ▲) or 1 mM EGTA (○, ●). Both preincubation and Mg^{2+}-ATPase reactions were carried out in the same buffer containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 60 mM KCl, 0.1 mM Ca^{2+}, or 1 mM EGTA.

gradient slab gel electrophoresis. Fodrin-containing fractions were pooled, concentrated, and stored at 4 °C (Figure 1). No myosin light chain kinase activity was detected in these preparations.

Electrophoreses. Electrophoresis was performed in 5–20% polyacrylamide gradient slab gels (1.5 mm thick) with a 4.5% acrylamide stacking gel, in the presence of 0.1% SDS using the buffer system according to Laemmli (1970). Electrophoresis through the stacking gel was performed at 25 mA and through the separating gel at 50 mA. Gels were stained in 40% (v/v) ethanol and 10% (v/v) acteic acid containing 0.06% Coomassie Brilliant Blue R-250 and destained in 20% (v/v) methanol and 10% (v/v) acetic acid.

Smooth Muscle Actomyosin Mg^{2+}-ATPase and Phosphorylation Assays. Routine assay was carried out in a reaction mixture containing actin, myosin, myosin light chain kinase, tropomyosin, and calmodulin at final concentrations of 0.25 mg/mL, 0.44 mg/mL, 10 μg/mL, 50 μg/mL, and 10 μg/mL, respectively. Reaction mixtures were preincubated at 30 °C for 2 min in buffer (20 mM Tris-HCl, pH 7.5, 60 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, and 0.1 mM $CaCl_2$) before initiation of the reaction by addition of [γ-^{32}P]ATP (specific activity 6000 cpm/nmol) to a final concentration of 1.0 mM. Aliquots were withdrawn at selected times (1, 2, 3, 4, 5, 7.5, and 10 min) for quantitation of [^{32}P]P_i release as previously described (Ikebe & Hartshorne, 1985). ATPase rates were calculated from the linear time course of ATP hydrolysis by linear regression analysis. The conditions for phosphorylation assays were the same as described for actomyosin Mg^{2+}-ATPase. [^{32}P]Phosphate incorporation into protein was quantitated as described by Walsh et al. (1983).

Preparation of Thiophosphorylated Myosin. Myosin was first diluted with buffer (10 mM Tris-HCl, pH 7.5, and 0.2 mM DTT) to a final concentration of 1.0 mg/mL and was thiophosphorylated for 10 min at 22 °C in 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM ATPγS in the presence of myosin light chain kinase (10 μg/mL) and calmodulin (10 μg/mL). The reaction mixture was then cooled on ice and slowly diluted with an equal volume of 2 mM EDTA, pH 7.5; 1 M $MgCl_2$ was then added dropwise to a final concentration of 15 mM to precipitate myosin. Myosin was sedimented by centrifugation at 15000*g* for 20 min. The pellet was dissolved in solubilization buffer containing 10 mM Tris-HCl, pH 7.5, 0.2 mM DTT, and 0.3 M KCl. The dilution–precipitation cycle was repeated 2 more times to remove calmodulin and myosin light chain kinase. The final pellet was dissolved in solubilization buffer and dialyzed overnight against two changes (4 L each) of the same buffer. Insoluble material was removed by centrifugation at 2000*g* for 5 min.

The thiophosphorylated myosin sample prepared by using this method was tested for calmodulin and myosin light chain kinase contaminations. An aliquot was incubated in boiling water for 2 min, and the denatured protein was removed by centrifugation at 2000*g* for 10 min. The supernatant was tested for the stimulation of calmodulin-dependent phosphodiesterase activity (Sharma et al., 1979). No calmodulin activity was detected. In the presence of calmodulin and myosin light chain kinase, the thiophosphorylated myosin could be phosphorylated by [γ-^{32}P]ATP to 0.2 mol of P_i/mol of thiophosphorylated myosin, suggesting that thiophosphorylation to the extent of 1.8 mol of P_i/mol of myosin was achieved. The omission of myosin light chain kinase from this mixture completely abolished the incorporation of [^{32}P]P_i into thiophosphorylated myosin, suggesting the complete removal of myosin light chain kinase by this purification method.

RESULTS

Fodrin Concentration and Preincubation Time Dependent Stimulation of Actomyosin Mg^{2+}-ATPase. The fact that fodrin is an actin binding protein suggests a potential role for fodrin in the regulation of cytoskeletal structure and/or motile processes. The possible involvement of fodrin in regulating the actin-activated Mg^{2+}-ATPase of smooth muscle myosin was examined in an in vitro system reconstituted from the purified contractile and regulatory proteins. Routine assay of actomyosin Mg^{2+}-ATPase involves incubation of reaction components in the presence of Ca^{2+} at 30 °C for 2 min prior to the addition of ATP to initiate the reaction. Figure 2a shows that when 3 nM fodrin was included in the reaction in the presence of Ca^{2+}, the Mg^{2+}-ATPase activity was stimulated by 23%. When the incubation time was increased to 10 min, fodrin-dependent stimulation was found to be 65% (Figure 2b). Similar results were obtained if Ca^{2+} instead of ATP was used to initiate the Mg^{2+}-ATPase reaction. The actomyosin

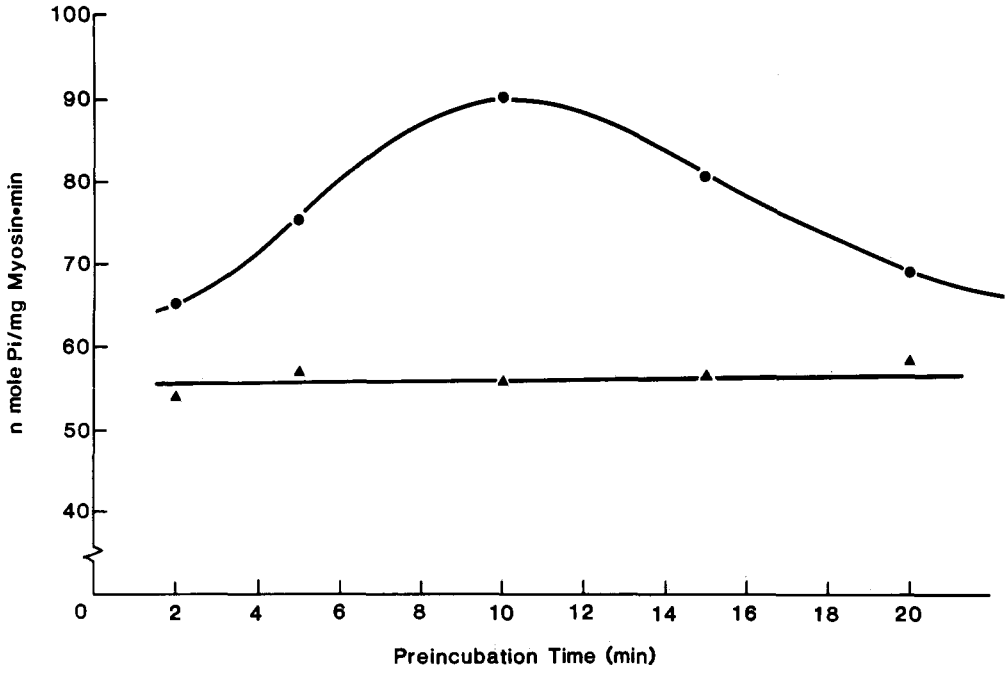

FIGURE 3: Dependence of fodrin stimulation of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity on preincubation time. Actin, myosin, myosin light chain kinase, tropomyosin, and calmodulin were preincubated at concentrations and conditions as indicated in Figure 2, in a Ca^{2+}-containing medium with (●) or without (▲) fodrin at 30 °C for 2, 5, 10, 15, and 20 min before initiation of the actomyosin Mg^{2+}-ATPase reaction by the addition of [γ-^{32}P]ATP. ATP rates were calculated from linear time course assays of ATP hydrolysis.

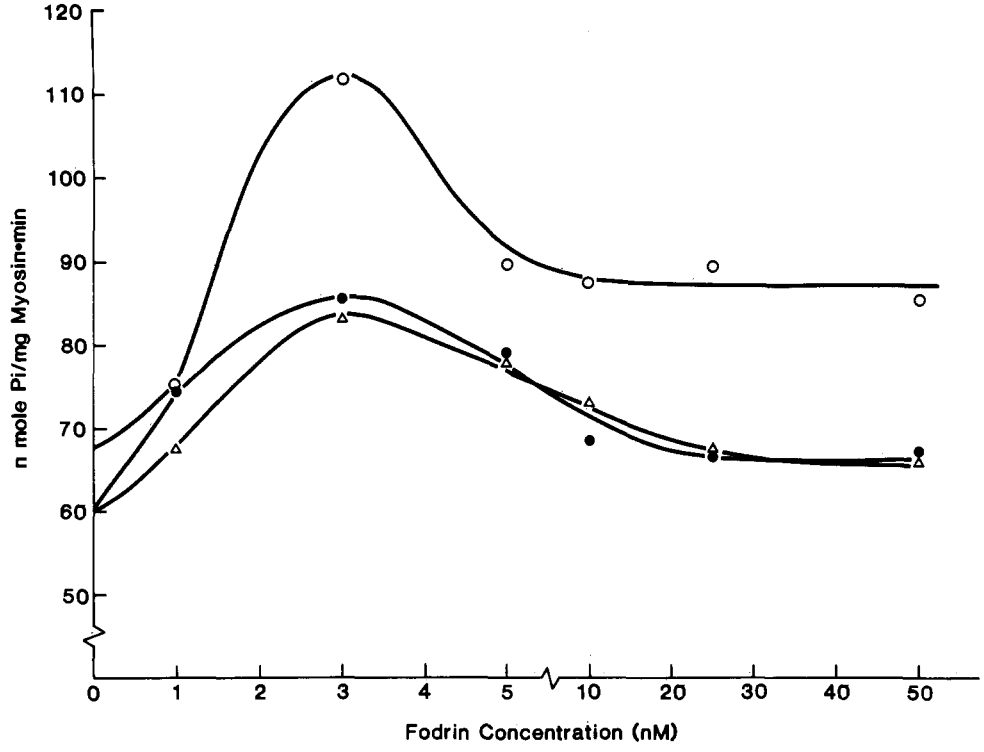

FIGURE 4: Effects of varying concentrations of fodrin on smooth muscle actin-activated myosin Mg^{2+}-ATPase activity. Bovine fodrin (1–50 μg/mL) was preincubated as described under Experimental Procedures in 0.1 mM Ca^{2+} at 30 °C for 5 (●), 10 (○), and 15 min (△), and the actin-activated myosin Mg^{2+}-ATPase rates were calculated from the linear time course assays.

Mg^{2+}-ATPase was essentially inactive irrespective of the presence or absence of fodrin when 0.1 mM Ca^{2+} was replaced by 1 mM EGTA (Figure 2). This result suggests that fodrin stimulation of Mg^{2+}-ATPase activity is also Ca^{2+} dependent. An alternative explanation is that fodrin is capable of stimulating phosphorylated but not unphosphorylated actomyosin Mg^{2+}-ATPase.

To characterize further this stimulation of Mg^{2+}-ATPase by fodrin, the effect of preincubation time was examined. Figure 3 shows that the stimulation of the actomyosin Mg^{2+}-ATPase exhibited a biphasic dependence on the time of incubation of the reaction components. Increase in the incubation time up to 10 min was accompanied by increased enzyme stimulation. Further incubation resulted in reversal of fodrin stimulation. In contrast to reactions containing fodrin, the Mg^{2+}-ATPase activities of control reactions showed no dependence on the time of preincubation (Figure 3). Subsequently, the dependence of fodrin stimulation of the actomyosin Mg^{2+}-ATPase on fodrin concentration was examined (Figure 4). At all preincubation times (5, 10, and 15

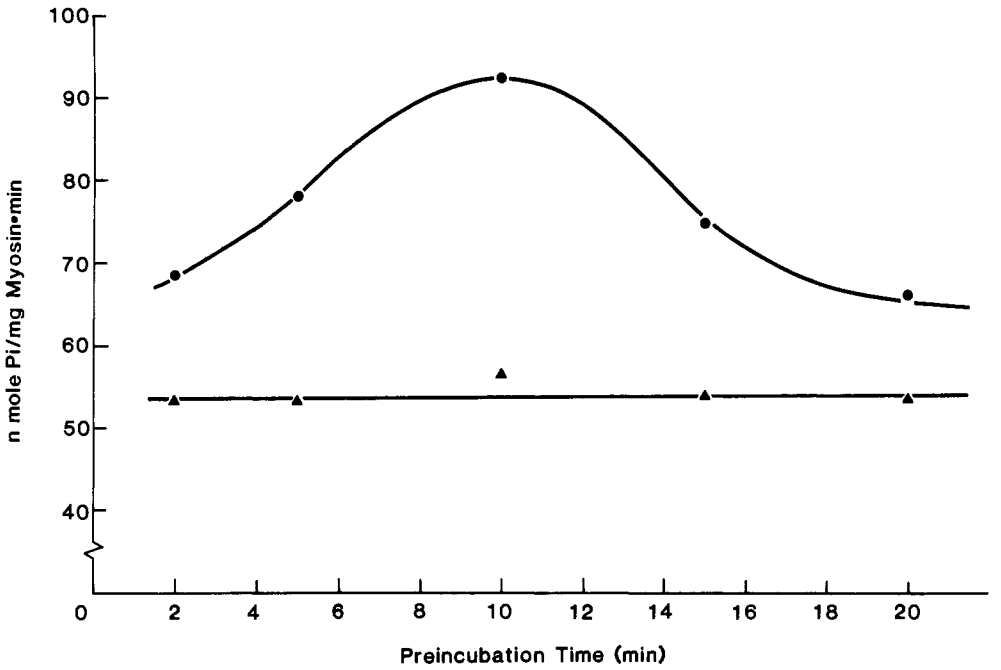

FIGURE 5: Dependence of fodrin stimulation of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity on actin and myosin preincubation. Actin and myosin were preincubated together at 30 °C for 2, 5, 10, 15, and 20 min separately from the remaining protein components. The other components of the actomyosin Mg^{2+}-ATPase system were then added with (●) or without (▲) fodrin prior to addition of [γ-^{32}P]ATP to start the reactions. ATPase rates were calculated from the linear time course assays.

min), the dependence of Mg^{2+}-ATPase stimulation on fodrin concentration was biphasic. Increase in fodrin concentration up to 3 nM (equivalent to a fodrin tetramer:actin monomer molar ratio of 1:1841) resulted in increased Mg^{2+}-ATPase stimulation, whereas beyond 3 nM the stimulatory effect was reversed. These results indicate that the preincubation time and fodrin concentration are two independent variables in the stimulation of actomyosin Mg^{2+}-ATPase. Although the maximal effect observed was only about 65%, both the magnitude of the effect and the conditions established for the demonstration of the effect are very reproducible (stimulation ranging from 50% to 70% using 15 different preparations of actin, myosin, and fodrin and 2 different methods of fodrin preparation; see Experimental Procedures).

Fodrin Stimulation Depends on the Preincubation of Actin and Myosin. To investigate the necessity for preincubation in more detail, various combinations of the contractile and regulatory proteins were preincubated for 10 min before measurement of the actomyosin Mg^{2+}-ATPase activity. The minimal protein components required for the stimulation were actin, myosin, and fodrin; successive addition of other protein components did not result in a significant increase in Mg^{2+}-ATPase activity (Table I). Further examination of various combinations of these three proteins in the preincubation mixture revealed that preincubation of actin and myosin alone was sufficient to result in stimulation by fodrin. Figure 5 shows that the dependence of Mg^{2+}-ATPase stimulation by fodrin on the preincubation time of actin and myosin is essentially the same as that of preincubation of the whole system (Figure 3). These observations suggest that fodrin stimulation requires a particular state of actomyosin which is achieved by a 10-min preincubation. The nature of this actomyosin state is unclear. However, various combinations of ionic conditions (60 or 100 mM KCl, 10 mM $MgCl_2$, and 0.1 mM Ca^{2+}) have been used in the preincubation and shown to have no significant effect on the fodrin stimulation of actomyosin Mg^{2+}-ATPase (Table II).

Fodrin Stimulation Depends on Tropomyosin. Action-activated myosin Mg^{2+}-ATPase activity requires tropomyosin for maximal activity (Sobieszek & Small, 1977). The effect of fodrin on Mg^{2+}-ATPase was examined in the absence and the presence of tropomyosin. In the absence of fodrin, the actomyosin rate [40.42 nmol of P_i (mg of myosin)$^{-1}$ min^{-1}] was approximately doubled by tropomyosin [74.74 nmol of

Table I: Proteins Required in Preincubation Mixture for Fodrin Stimulation of Actin-Activated Smooth Muscle Myosin Mg^{2+}-ATPase Activity

preincubation mixture[a]	% activation over control[b]
A + F	12.0
M + F	18.0
A + M + F	53.6
A + M + TM + F	52.4
A + M + TM + MLCK + F	59.7
A + M + TM + MLCK + CaM + Ca^{2+} + F	53.5

[a] The indicated proteins were incubated in the presence of 20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM DTT for 10 min at 30 °C prior to the addition of the remaining components of the Mg^{2+}-ATPase reaction. The final mixtures which contained actin, myosin, MLCK, TM, CaM, and fodrin in 20 mM Tris-HCl, pH 7.5, 60 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, and 0.1 mM Ca^{2+} were incubated at 30 °C for another 2 min before the initiation of the ATPase reaction. [b] The percent activation was calculated relative to the rate of control actomyosin Mg^{2+}-ATPase activity (no fodrin added). This rate was 57.6 nmol of P_i (mg of myosin)$^{-1}$ min^{-1}.

Table II: Effect of Ionic Conditions in the Actin–Myosin Preincubation Mixture on Fodrin Stimulation of Actin-Activated Smooth Muscle Myosin Mg^{2+}-ATPase Activity[a]

ionic conditions	% activation over control[b]
100 mM KCl	59.9
60 mM KCl	52.4
60 mM KCl + 0.1 mM Ca^{2+}	52.7
60 mM KCl + 10 mM $MgCl_2$	59.8
60 mM KCl + 0.1 mM Ca^{2+} + $MgCl_2$	61.7

[a] Actin and myosin were preincubated in the presence of 20 mM Tris, pH 7.5, and 1 mM DTT under the indicated ionic conditions for 10 min at 30 °C. Actomyosin Mg^{2+}-ATPase activities were measured as described in Table I. [b] The rate of control actomyosin Mg^{2+}-ATPase activity was 62.2 nmol of P_i (mg of myosin)$^{-1}$ min^{-1}.

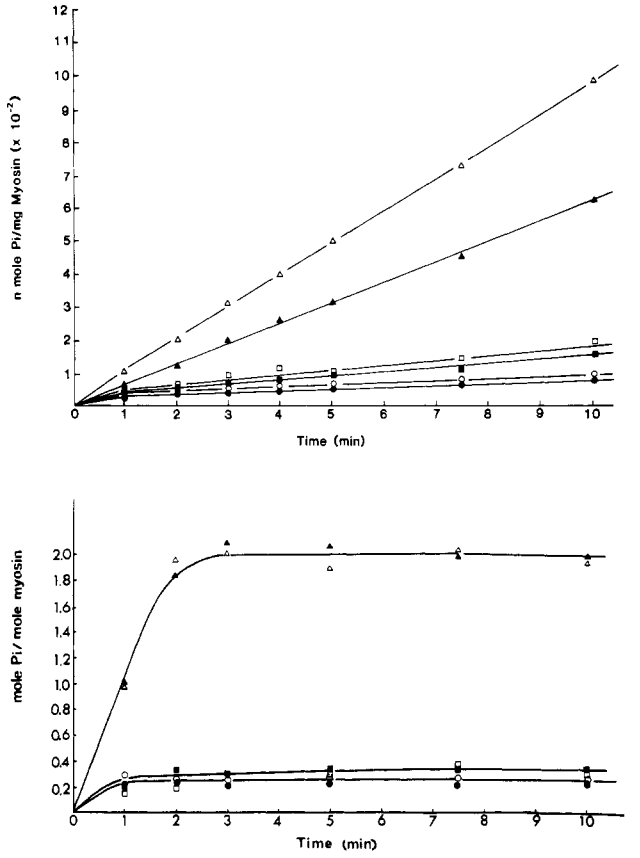

FIGURE 6: Relationship between fodrin stimulation and myosin light chain kinase activation of actin-activated myosin Mg^{2+}-ATPase activity. (Top) Mg^{2+}-ATPase activities were measured as described under Experimental Procedures after preincubation for 10 min at 30 °C with (△, ○) or without (▲, ●) fodrin in the presence of 0.1 mM Ca^{2+} (△, ▲) or 1 mM EGTA (○, ●). The experiment was also performed in the absence of myosin light chain kinase and the presence of 0.1 mM Ca^{2+} with (□) or without (■) fodrin. (Bottom) Mg^{2+}-ATPase reactions preincubated for 10 min with (△, □, ○) or without (▲, ■, ●) 3 nM fodrin were carried out as described in the top panel. At different time intervals, aliquots (250 μL) were removed, and phosphate incorporation was determined (see Experimental Procedures).

P_i (mg of myosin) $^{-1}$ min^{-1}]. Fodrin stimulation was completely abolished in the absence of tropomyosin [34.70 nmol of P_i (mg of myosin)$^{-1}$ min^{-1}]. The stimulation was restored in the presence of tropomyosin [120.35 nmol of P_i (mg of myosin)$^{-1}$ min^{-1}]. The Mg^{2+}-ATPase rate in the absence of fodrin as well as the extent of fodrin stimulation was not changed when the tropomyosin concentration was doubled. These results suggest that the effect of fodrin on the actin-activated Mg^{2+}-ATPase can be observed only when the system is fully activated. The Mg^{2+}-ATPase rates were calculated from the linear time course assays with linear regression values (r) ranging from 0.993 to 1.000.

Relationship between Fodrin Stimulation and Myosin Phosphorylation. To test whether or not fodrin could stimulate actomyosin Mg^{2+}-ATPase activity in the absence of phosphorylation of LC20, the effect of fodrin on actomyosin Mg^{2+}-ATPase activity in the absence of added MLCK was examined (Figure 6, top). In this case, the Mg^{2+}-ATPase showed very low activity either in the absence or in the presence of fodrin, suggesting that fodrin stimulated the phosphorylated but not the nonphosphorylated actomyosin Mg^{2+}-ATPase. The control sample (no fodrin added) showed slightly higher activity in the presence of Ca^{2+} than in the presence of EGTA. This Ca^{2+}-dependent activity could be attributed to trace contamination of the myosin preparation with MLCK. Quantification of phosphate incorporation into myosin revealed that, in the presence of MLCK and Ca^{2+}, myosin was rapidly phosphorylated to the extent of 2.0 mol of phosphate/mol of myosin (Figure 6, bottom), whereas in the absence of added MLCK, or in the presence of MLCK and EGTA, myosin was phosphorylated to the extent of 0.28 or 0.21 mol of phosphate/mol of myosin, respectively. In all cases, fodrin was found to have no effect on both the rates and the extents of phosphorylation (Figure 6, bottom). Analysis of the protein samples by SDS–PAGE followed by autoradiography revealed that irrespective of the presence of fodrin, the 20 000-dalton light chain was the only polypeptide phosphorylated (data not shown). The result suggests that fodrin stimulation is not due to an effect on protein phosphorylation.

Fodrin Stimulation Is Ca^{2+} and Calmodulin Dependent. Since MLCK reaction depends on Ca^{2+} and calmodulin and since fodrin stimulation of actomyosin Mg^{2+}-ATPase appears to require the phosphorylation of myosin, it is not feasible to determine the dependence on Ca^{2+} and calmodulin of fodrin stimulation in a normal actomyosin Mg^{2+}-ATPase reaction. It has been shown that myosin can be irreversibly thiophosphorylated and locked in an active state which no longer requires Ca^{2+} or calmodulin for its actomyosin Mg^{2+}-ATPase activity (Hoar et al., 1979). We have therefore used thiophosphorylated myosin to study the involvement of calmodulin in fodrin stimulation. Smooth muscle myosin was thiophosphorylated using Ca^{2+}, calmodulin, MLCK, and MgATPγS, and thiophosphorylated myosin was purified free of calmodulin and MLCK as described under Experimental Procedures. When reconstituted with actin and tropomyosin, this activated myosin no longer required MLCK or Ca^{2+} and calmodulin for activity (Figure 7). Under this condition, fodrin was able to stimulate the acto–thiophosphorylated myosin Mg^{2+}-ATPase only if both Ca^{2+} and calmodulin were present (Figure 7). The result indicates that fodrin stimulation is Ca^{2+} and calmodulin dependent and substantiates the suggestion that fodrin stimulates the phosphorylated, not the nonphosphorylated, myosin.

Discussion

The present results clearly establish that bovine fodrin is capable of stimulating actomyosin Mg^{2+}-ATPase activity in a system reconstituted from purified rabbit skeletal muscle actin and chicken gizzard smooth muscle myosin and regulatory proteins. Although maximal stimulation by fodrin is only 50–70% over that of actin-activated myosin Mg^{2+}-ATPase activity, both the extent of stimulation and the conditions required for the effect of stimulation are reproducible. Others (Shimo-Oka & Wananabe, 1980; Wagner, 1984) have shown that skeletal actomyosin Mg^{2+}-ATPase activity could be activated by fodrin under certain conditions. Wagner (1984) carried out detailed characterization of the stimulation of skeletal muscle actomyosin Mg^{2+}-ATPase and found that stimulation was observed when fodrin was added to the actin and myosin reaction mixture prior to the addition of ATP. If ATP was added a few minutes before the addition of fodrin, inhibition rather than activation of the Mg^{2+}-ATPase by fodrin was observed. Both the inhibition and activation of skeletal muscle actomyosin Mg^{2+}-ATPase appeared to be Ca^{2+} dependent, although the source of Ca^{2+} sensitivity in this system is not known. With the reconstituted system used in the present study, only fodrin stimulation could be demonstrated, and the stimulation was not affected by the preincubation of the contractile and regulatory proteins with ATP. This stimulation is Ca^{2+} dependent as well as calmodulin dependent. Although fodrin has been shown to affect the polymerization

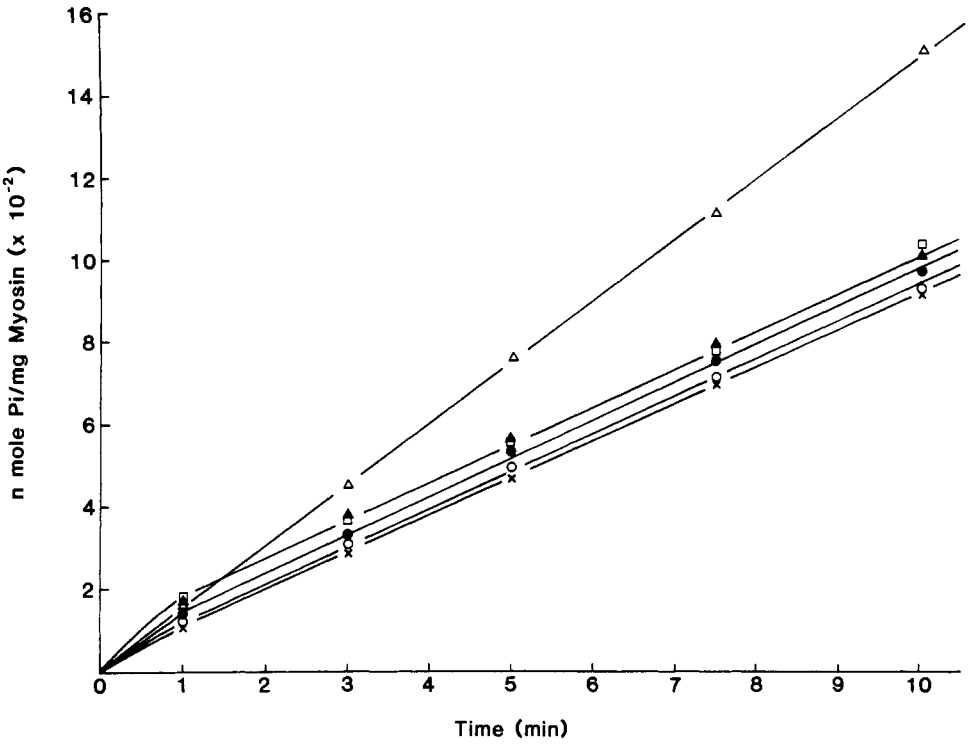

FIGURE 7: Ca^{2+}- and calmodulin-dependent stimulation of actin-activated thiophosphorylated myosin Mg^{2+}-ATPase activity by fodrin. Myosin was thiophosphorylated and purified free of calmodulin and myosin light chain kinase as described under Experimental Procedures. Thiophosphorylated myosin was incubated with actin and tropomyosin in the presence of Ca^{2+} (○, ▲), Ca^{2+} and calmodulin (□, △), and calmodulin and EGTA (×, ●) in the presence (▲, △, ●) or absence (○, □, ×) of fodrin.

of G-actin (Sobue et al., 1982) and the cross-linking of F-actin (Glenney et al., 1982), the present results represent the first demonstration of the dependence of a fodrin effect on Ca^{2+} and calmodulin.

The stimulation of smooth muscle actomyosin Mg^{2+}-ATPase by fodrin appears to be highly complex and dependent on specific conditions. Many aspects of this stimulation are not fully understood at present. The dependence of Mg^{2+}-ATPase stimulation on fodrin concentrations shows a biphasic curve. One possible explanation for this phenomenon may be that fodrin could bind at distinct sites on actin with different affinities, binding of fodrin at the high-affinity sites causing an increase in Mg^{2+}-ATPase activity whereas binding at low-affinity sites reversed the stimulation. Clearly, there are many other possible explanations for the observation. The low concentration of fodrin (molar ratio of fodrin tetramer:actin monomer = 1:1841) required for optimal effect has also raised the possibility that the protein may act catalytically. There are at least four other requirements in addition to the critical fodrin concentration required for actomyosin Mg^{2+}-ATPase stimulation: (i) The myosin molecule has to be in the active state. Fodrin stimulation was observed when MLCK was present simultaneously in the reaction mixture, or if myosin had been previously thiophosphorylated. (ii) The incubation time of actin and myosin prior to the initiation of the Mg^{2+}-ATPase is critical. The observation suggests that actin and myosin undergo changes during the incubation and a transitory form of the protein complex, reached at 10-min preincubation, is susceptible to fodrin stimulation. Since the Mg^{2+}-ATPase activity measured in the absence of fodrin does not change significantly upon preincubation (Figures 3 and 5), this transitory form of actomyosin has not been previously detected. (iii) Fodrin stimulation of actin-activated myosin Mg^{2+}-ATPase absolutely depends on tropomyosin. (iv) Fodrin stimulation is Ca^{2+} and calmodulin dependent. Results from other investigators (Kakiuchi et al., 1982; Glenney et al., 1982) show that the binding of fodrin to actin filaments is not dependent on Ca^{2+} and calmodulin. This, it appears that Ca^{2+} and calmodulin may affect the interaction between fodrin and actin in a more subtle way. Although fodrin can bind to and cross-link F-actin (Glenney et al., 1982), the observation of fodrin stimulation of smooth muscle actomyosin Mg^{2+}-ATPase is probably not due to F-actin cross-linking on the basis of two criteria: (a) The F-actin cross-linking is not affected by Ca^{2+} and calmodulin (Glenney et al., 1982), and (b) the concentration of fodrin in our experiments is much lower than the amount of fodrin required to cause F-actin cross-linking (Burridge et al., 1982). Physiological studies as described in the introduction suggest that there might exist a secondary Ca^{2+}-dependent mechanism in the regulation of actin and myosin interaction in smooth muscle. The Ca^{2+}- and calmodulin-dependent stimulation of actomyosin Mg^{2+}-ATPase by fodrin may be considered as a possible mechanism.

In conclusion, we have shown that fodrin is capable of stimulating smooth muscle actomyosin Mg^{2+}-ATPase in a Ca^{2+}- and calmodulin-dependent manner and established the conditions under which this stimulation could be consistently observed. We suggest that the stimulation by fodrin on smooth muscle actomyosin Mg^{2+}-ATPase is physiological. We have surveyed a variety of smooth muscle and nonmuscle tissues including chicken gizzard (Wang et al., unpublished data) and found that fodrin is widely distributed among tissues. This result agrees with those reported by other investigators (Goodman & Zagon, 1984). Although smooth and nonmuscle myosins are not identical, it is generally believed that they share some structural and regulatory properties (Adelstein & Esienberg, 1980). It is tempting to suggest that the observed effects of fodrin on smooth muscle actonmyosin Mg^{2+}-ATPase may also occur in nonmuscle cells.

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Registry No. ATPase, 9000-83-3; Ca, 7440-70-2.

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Prothrombin Tokushima, a Replacement of Arginine-418 by Tryptophan That Impairs the Fibrinogen Clotting Activity of Derived Thrombin Tokushima†

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ABSTRACT: Structural studies on a hereditarily abnormal prothrombin, prothrombin Tokushima, have been performed to identify the difference responsible for its reduced fibrinogen clotting activity upon conversion to thrombin. The prothrombin sample used was from a heterozygote but contained exclusively a defective prothrombin molecule, since the patient was heterozygous for both dysprothrombinemia and hypoprothrombinemia. Amino acid sequence analysis of a peptide isolated from a lysyl endopeptidase digest of the abnormal thrombin indicated that Arg-418 (equivalent to Asn-101 in the chymotrypsin numbering system) had been replaced by Trp. This amino acid substitution can result from a single nucleotide change in the codon for Arg-418 (CGG → TGG). The Arg → Trp replacement found in the thrombin portion of prothrombin Tokushima appears to reduce its interaction with various substrates including fibrinogen and platelet receptors and accounts for the recurrent bleeding episode observed in the propositus.

Human prothrombin is a single-chain glycoprotein composed of 579 amino acid residues (Walz et al., 1977; Butokowski et al., 1977; Thompson et al., 1977; Magnusson et al., 1975; Degen et al., 1983). This protein can be divided into three segments, fragment 1 (residues 1–155), fragment 2 (residues 156–271), and prethrombin 2 (residues 272–579). Upon activation by factor Xa, prethrombin 2 is cleaved at an Arg–Ile linkage between residues 320 and 321, resulting in the formation of two-chain α-thrombin (Downing et al., 1975). Human α-thrombin is composed of a small A chain (residues 285–320) and a large B chain (residues 321–579), and these

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