Roles of Calcium and Phosphorylation in the Regulation of the Activity of Gizzard Myosin†

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ABSTRACT: In the theory of regulation of smooth-muscle actomyosin involving phosphorylation, it is generally accepted that the Mg^{2+}-ATPase activity of phosphorylated myosin is activated by actin, whereas the activity of the unphosphorylated myosin is not. The role of Ca^{2+}, however, is not clear and here there are basically two possibilities; i.e., either Ca^{2+} acts only to activate the myosin light-chain kinase or, that in addition to the sites on the myosin light-chain kinase, other regulatory sites are involved, presumably on the myosin molecule. To distinguish between these possibilities, three types of experiments were performed: (1) Gizzard myosin was phosphorylated and then separated from the myosin light-chain kinase and phosphatase by chromatography on Sepharose 4-B. The actin-activated Mg^{2+}-ATPase activity of this stable phosphorylated myosin was not inhibited by the removal of Ca^{2+}. (2) Adenosine 5′-*O*-(3-thiotriphosphate) was used as a substrate for the myosin light-chain kinase. The resultant thiophosphorylated myosin is resistant to hydrolysis by the phosphatase and, therefore, the myosin was trapped in the thiophosphorylated state. It was found that as the proportion of thiophosphorylated myosin increased the inhibition in the absence of Ca^{2+} of the actin-activated ATPase activity decreased; i.e., Ca^{2+} sensitivity was progressively lost. (3) The rate of inhibition of the actin-activated ATPase activity of phosphorylated myosin (in a system containing kinase and phosphatase) following the removal of Ca^{2+} was measured and found to correlate well with the rate of removal of phosphate groups from the myosin light chains. It is concluded that the dominant role of Ca^{2+} is to activate the myosin light-chain kinase. No evidence for the implication of additional regulatory sites was obtained, and thus the actin-activated ATPase activity of gizzard myosin is dependent only on its state of phosphorylation. It follows that the actin-activated ATPase activity of phosphorylated myosin is not inhibited directly as a result of the removal of Ca^{2+} but is inhibited by the removal of the phosphate groups from the myosin light chains.

It is generally accepted that in smooth as in skeletal muscle the development of tension and shortening is due to the interaction of the myosin cross-bridges with actin and the associated hydrolysis of ATP. It is also accepted that the myosin–actin contacts are initiated as a result of an increase in the intracellular concentration of ionized Ca^{2+}. However, there is no universal agreement on the regulatory mechanism that controls these interactions. In skeletal muscle the regulatory proteins are troponin and tropomyosin which are located on the thin filament. It has been suggested (see review, Weber and Murray, 1973) that these proteins function by allowing the interaction of the myosin cross-bridges with actin only in the presence of Ca^{2+}. It is reasonable to expect that in smooth muscle the regulatory proteins fulfill a similar role, although there is one basic difference that must be emphasized: namely, that the Mg^{2+}-ATPase activity of pure skeletal muscle myosin and pure skeletal muscle actin is close to maximal, whereas the equivalent mixture from smooth muscle has negligible Mg^{2+}-ATPase activity (Bárány et al., 1966; Yamaguchi et al., 1970). Thus, the regulatory mechanism in skeletal muscle operates by inhibiting an active state in the absence of Ca^{2+}, whereas in smooth muscle it operates by activating a dormant state in the presence of Ca^{2+}. This basic difference has been stressed repeatedly by Ebashi and his co-workers (Ebashi et al., 1975a,b; Ebashi et al., 1976). It has a practical significance in that the search for regulatory proteins in smooth muscle should be focused initially on Ca^{2+}-dependent activators.

There are basically three theories which have been proposed to account for regulation in smooth muscle. These are: (1) that a troponin-like mechanism exists (Ebashi et al., 1966; Carsten, 1971; Ito and Hotta, 1976; Ito et al., 1976; Head et al., 1977), (2) that regulation is achieved via phosphorylation of the 20 000-dalton light chains of myosin and thus requires a myosin light-chain kinase and phosphatase, and (3) that control is mediated via an 80 000-dalton protein (Mikawa et al., 1977a,b; Hirata et al., 1977) whose mode of action is unknown but is thought not to involve the phosphorylation of myosin. [The earlier suggestion by Ebashi et al. (1966) of a troponin-like system in smooth muscle has been withdrawn (Mikawa et al., 1977b)].

The most popular of the three proposed mechanisms is the concept that regulation in smooth muscle is dependent upon the state of phosphorylation of myosin. This was shown first by Sobieszek (1977a) and has subsequently been described by several investigators (Aksoy et al., 1976; Chacko et al., 1977; Sobieszek and Small, 1977; Ikebe et al., 1977; Sobieszek, 1977b). Recently it has been found that the myosin light-chain kinase is composed of two subunits of approximate molecular weights 105 000 and 17 000 (Dąbrowska et al., 1977). The smaller subunit appears to be identical with the phosphodiesterase activator or modulator protein (Dąbrowska et al., 1978). In the latter studies, we found a consistent relationship between the phosphorylation of myosin and the actin activation of Mg^{2+}-ATPase activity. Since these results were obtained using purified components, the possibility of the regulatory factor being a contaminant of the kinase preparations was considered

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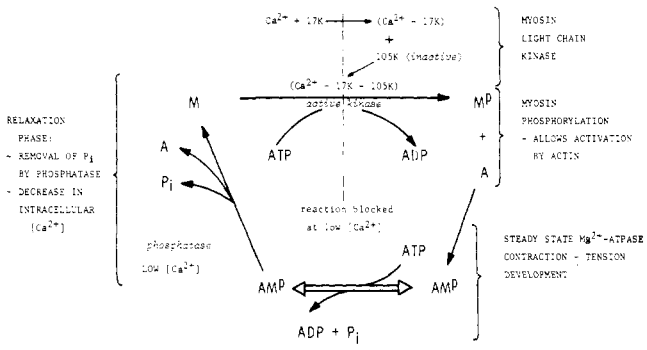

FIGURE 1: Schematic representation of the role of phosphorylation in the regulatory mechanism in smooth muscle. Abbreviations used are: M, myosin; A, actin; 105K and 17K, the 105 000- and 17 000-dalton components of the myosin light-chain kinase, respectively; P_i, inorganic phosphate; M^P and AM^P, phosphorylated myosin and actomyosin, respectively. The double arrow at the bottom of the diagram indicates that phosphorylated myosin will hydrolyze (as actomyosin) several molecules of ATP before the phosphatase removes the phosphate groups from the myosin light chains; i.e., the turnover of ATP by the active site of phosphorylated actomyosin is faster than that of the kinase–phosphatase system.

unlikely, and therefore the phosphorylation scheme was accepted with more confidence. The critical points in this scheme are: (a) phosphorylation of the 20 000-dalton light chains of myosin is catalyzed by an ATP–myosin light-chain phosphotransferase (i.e., the kinase) in the presence of Ca^{2+} but not in its absence; (b) phosphorylation allows the subsequent activation by actin of the myosin Mg^{2+}-ATPase activity (Górecka et al., 1976); (c) in the absence of Ca^{2+} the phosphate groups are removed by a phosphatase, and the hydrolysis of ATP ceases. The mechanism may be represented in a schematic form as shown in Figure 1. Although there are several aspects of this cycle that need to be investigated further, in this communication we will concentrate on only one part, namely, the role of Ca^{2+} in the relaxation phase. The simplest mechanism to account for relaxation is that removal of Ca^{2+} inactivates the kinase and allows the phosphatase to release the phosphate groups from the myosin light chains. The nonphosphorylated myosin is not activated by actin and thus the muscle relaxes. In this theory, the dominant feature is whether or not the myosin is phosphorylated, and the only function of Ca^{2+} is to regulate the activity of the kinase. This is the view held by Small and Sobieszek (1977a,b). However, it is known that smooth-muscle myosin binds Ca^{2+} (Hartshorne et al., 1977a; Sobieszek and Small, 1976), and it has been suggested (Chacko et al., 1977) that the Ca^{2+}–myosin interaction is an important feature of the regulatory mechanism in smooth muscle. This hypothesis is made more attractive by the demonstration (Szent-Györgyi et al., 1973; Lehman and Szent-Györgyi, 1975; Kendrick-Jones et al., 1976) that in several of the lower phyla regulation is achieved solely by the interaction of Ca^{2+} with the myosin light chains. Thus, the question that we have posed is whether or not the binding of Ca^{2+} to myosin has any role in the regulatory mechanism of smooth muscle.

The simplest way to resolve the problem would be to prepare phosphorylated myosin and measure the actin-activated ATPase activity in the presence and absence of Ca^{2+}. If it is essential for ATPase activity to have Ca^{2+} bound to myosin, then in the absence of Ca^{2+} the phosphorylated myosin would not be activated by actin. If, on the other hand, the Ca^{2+}–myosin interaction is not critical and the dominant feature is whether or not the myosin is phosphorylated, then the ATPase activity would not be inhibited at low Ca^{2+} concentrations. In order to do this experiment, however, it is necessary to prepare phosphorylated myosin free of phosphatase and kinase activity. In our experience this proved to be difficult to do, and, although a stable phosphorylation level in the absence of Ca^{2+} was obtained (i.e., an indication of the lack of phosphatase), we were unable to achieve higher than 50% of the saturation level of phosphorylation (see Results). The indication from these studies was that Ca^{2+} binding to myosin was not a feature of the regulatory mechanism, but in order to confirm this we felt that alternative approaches were necessary, and two experiments were devised. These were: (a) the use of ATPγS[1] (Goody and Eckstein, 1971) as a substrate for the myosin light-chain kinase and (b) a study of the rate of inhibition of the Mg^{2+}-ATPase activity of actomyosin following the addition of EGTA.[1]

The rationale behind the first experiment is that it has been shown that ATPγS serves as a substrate for several protein kinases (Goody et al., 1972; Perry et al., 1975), but, in general, the thiophosphorylated protein is not a favored substrate for the phosphatase (Gratecos and Fischer, 1974; Gergely et al., 1976; Morgan et al., 1976), and thus the protein is trapped in the phosphorylated state. Clearly, if this situation were applied to the smooth-muscle system it would provide us with a stable thiophosphorylated myosin, and this would allow the subsequent evaluation of myosin phosphorylation vs. Ca^{2+} binding by myosin as components of the regulatory mechanism.

In the second approach the objective was to establish the kinetics of the various events that occurred following the removal of Ca^{2+} (i.e., following the addition of EGTA). The experiment was to establish the Mg^{2+}-ATPase rate for actomyosin in the presence of Ca^{2+}, to then add EGTA, and determine the rate at which the inhibited state was attained and to correlate this with the rate of phosphate removal. It was reasoned that if Ca^{2+} removal from myosin determines the kinetics of ATPase inhibition then the inhibited state would be attained rapidly. For example, with troponin C the removal of Ca^{2+} from the Ca^{2+}-specific sites has a $t_{1/2}$ of approximately 3 ms, and the removal of Ca^{2+} from the Ca^{2+}–Mg^{2+} sites a $t_{1/2}$ of approximately 700 ms (Johnson et al., 1978). On the other hand, if the extent of inhibition is a reflection of the level of phosphorylation of myosin, then the rate at which the inhibited state is attained should correlate with the phosphatase rate.

The results from each type of experiment are consistent and suggest that the interaction of Ca^{2+} with myosin does not play a dominant role in the regulatory mechanism of smooth muscle. The important feature is the phosphorylation of myosin, and, in our opinion, the Ca^{2+} sensitivity of the mechanism is due entirely to the interactions of Ca^{2+} with the myosin light-chain kinase.

Experimental Procedure

Protein Preparations. Myosin was isolated from frozen chicken gizzards (Pel Freez Biologicals, Rogers, Ark.) as described by Hartshorne et al. (1977b). Actin and tropomyosin were prepared from rabbit skeletal muscle by the procedure of Driska and Hartshorne (1975), and troponin was isolated using the method of Hartshorne and Mueller (1969). The native tropomyosin preparation from chicken gizzards (Ebashi et al., 1966) was used as the source of myosin light-chain kinase and phosphatase. This fraction, without further purification, is referred to as the crude kinase. The major component of the

[1] Abbreviations used: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD.

crude kinase was tropomyosin. In some experiments the kinase following the removal of tropomyosin was used. The tropomyosin-free kinase was obtained as the supernatant following isoelectric precipitation at pH 4.8 in 0.2 M KCl, 0.2 mM dithiothreitol. This will be referred to as the kinase supernatant.

The stable phosphorylated myosin (i.e., phosphorylated myosin separated from the kinase and phosphatase) was prepared essentially as described by Adelstein et al. (1976) as follows: Gizzard myosin was phosphorylated using γ-labeled [^{32}P]ATP in the presence of Ca^{2+} and excess myosin light-chain kinase, under the solvent conditions used for ATPase assays. After 5 min at 25 °C, KCl and potassium phosphate (pH 7.5) were added to final concentrations of 1 and 0.1 M, respectively. The mixture was cooled to 4 °C and applied to a Sepharose 4-B column (2.5 × 90 cm) equilibrated with 0.8 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 0.5 mM ATP. The elution of protein was detected by adding 0.2-mL aliquots of each fraction to 0.8 mL of 12.5% trichloroacetic acid and measuring the A_{320nm}. The myosin-containing fractions (the elution position of myosin was determined previously using $NaDodSO_4$-polyacrylamide electrophoresis) were assayed for ^{32}P content and actin-activated ATPase activity. At high ionic strength, for example, in the chromatography solvent, the protein-bound ^{32}P was stable over a period of several days, in agreement with the results of Chacko et al. (1977). However, at lower ionic strength, for example, in the ATPase assay solutions, some dephosphorylation was detected over a period of a few hours. Therefore, the column fractions were assayed directly, avoiding dialysis. In order to minimize the amount of protein required and also to maintain the final ionic strength of the assay solutions at less than $I \simeq 0.08$, an assay temperature of 37 °C was used.

Assay Procedures. ATPase and phosphorylation assays were performed in 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris-HCl (pH 7.5). EGTA (1 mM) was added when it was required to perform assays in the absence of Ca^{2+}. P_i liberation was assayed as described previously (Driska and Hartshorne, 1975). ADP production was monitored at constant ATP concentrations, maintained via the enzymatic coupling to NADH oxidation, in two ways. In the first method, phosphoenolpyruvate (2.85 mM), pyruvate kinase (20 units/mL), lactate dehydrogenase (29 units/mL), and NADH (0.9 mM) were added to the ATPase assay solvent described above. At various times 0.2-mL aliquots of the assay mixture were removed and added to 0.5 mL of 4% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.5). The extent of ADP hydrolysis was calculated directly from the conversion of NADH to NAD^+ using an E_{340nm} of 6.22×10^3. In the second method, the conversion of NADH to NAD^+ was monitored continuously using an Aminco DW-2 spectrophotometer in the presence of phosphoenolpyruvate (2.0 mM), pyruvate kinase (70 units/mL), lactate dehydrogenase (100 units/mL), and NADH (0.56 mM). Other conditions were as above, except for the ATP concentration which was reduced to 1.66 mM. Monitoring changes in the difference between A_{340nm} and A_{420nm} eliminated complications arising from sample turbidity and superprecipitation.

Phosphorylation assays were carried out as described by Aksoy et al. (1976) with the following modifications. A protein carrier, bovine serum albumin, approximately 2 mg/assay, was added to samples immediately preceding the addition of trichloroacetic acid to facilitate the quantitative transfer of phosphorylated protein to the Millipore filters. Following exhaustive rinsing of the precipitates with 5% trichloroacetic acid and 1% $Na_4P_2O_4$, the precipitates were rinsed with 3 mL of anhydrous ether. Fifteen percent paraffin in cyclohexane (0.2 mL) was applied to each filter to serve as an adhesive prior to the transfer to the scintillation vials.

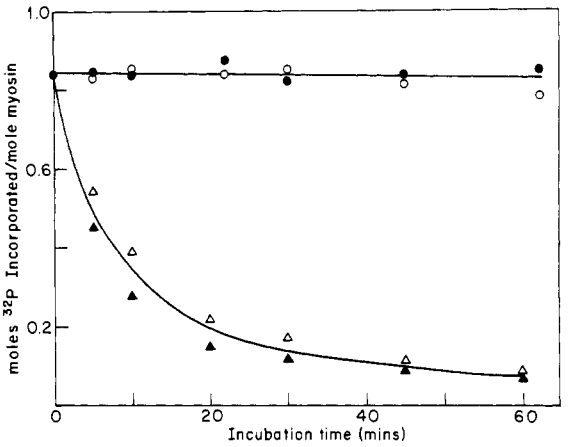

FIGURE 2: The stability of phosphorylated gizzard myosin isolated by Sepharose 4-B chromatography and the effect of added kinase-phosphatase on the phosphorylation level. The myosin was phosphorylated and isolated on Sepharose 4-B as described under Experimental Procedure. The phosphorylated myosin (0.04 mg/mL) was incubated in 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris-HCl (pH 7.5) at 37 °C, and the ^{32}P levels were determined at the times indicated. Incubation was carried out in the absence (●) and presence (○) of 1 mM EGTA. To an identical sample, crude kinase (ca. 0.02 mg/mL) was added and the ^{32}P levels were determined following incubation in the absence (▲) and presence (△) of 1 mM EGTA.

Gel Electrophoresis. $NaDodSO_4$-polyacrylamide electrophoresis was carried out at 5.6% acrylamide under the conditions of Fairbanks et al. (1971). Electrophoresis in polyacrylamide gels and 7 M urea was basically as described by Perrie and Perry (1970), except that the protein prior to electrophoresis was dialyzed exhaustively against 8 M urea, 33 mM Tris-glycine (pH 8.6), 0.17 mM EDTA, and 16 mM β-mercaptoethanol, and a spacer gel (3.5% acrylamide) approximately 7 mm, was used on top of the running gel. These modifications were suggested by Siemankowski and Dreizen (1978).

Materials. ATPγS was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and [γ-^{32}P]ATP was obtained from New England Nuclear, Boston, Mass. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo.

Results

Stable Phosphorylated Myosin. Gizzard myosin was phosphorylated using [γ-^{32}P]ATP and excess myosin light-chain kinase and subjected to Sepharose 4-B chromatography at high ionic strength (see Experimental Procedure). The object was to isolate phosphorylated myosin and in particular to remove contamination by the phosphatase and kinase. During the chromatographic fractionation the action of the phosphatase was prevented by using a high ionic strength solvent, $I > 0.8$ (Chacko et al., 1977). The ^{32}P content of the chromatographically purified myosin was determined and found to be 0.84 mol of P/mol of myosin, i.e., representing only 42% of the maximal or saturation level. In two other similar experiments, the level of phosphorylation did not exceed 1 mol of P/mol of myosin. The submaximal level of phosphorylation was, however, reasonably stable when assayed under the solvent conditions used for ATPase assays as shown in Figure 2, over a 60-min exposure. This observation was critical as it al-

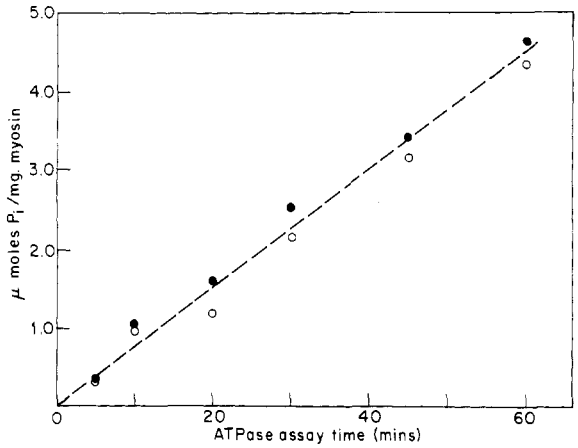

FIGURE 3: The actin-activated ATPase activity of the phosphorylated gizzard myosin isolated by Sepharose 4-B chromatography. The ATPase assay conditions were 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris-HCl (pH 7.5), 37 °C, myosin (0.04 mg/mL), skeletal actin (0.39 mg/mL), and skeletal tropomyosin (0.13 mg/mL). Assays were carried out in the absence (●) and presence (○) of 1 mM EGTA.

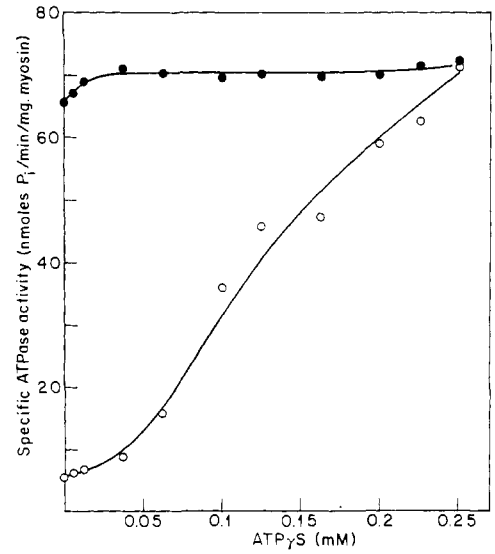

FIGURE 4: The effect of ATPγS on the actin-activated ATPase activity of gizzard myosin measured in the presence and absence of Ca^{2+}. Gizzard myosin (0.45 mg/mL) was preincubated with the indicated levels of ATPγS in 50 mM KCl, 10 mM $MgCl_2$, and 25 mM Tris-HCl (pH 7.5) in the presence of crude kinase (0.3 mg/mL) and skeletal actin (0.2 mg/mL). After 15 min at 25 °C, either ATP or ATP plus EGTA were added, and the ATPase activity was determined. The final concentrations of ATP and EGTA were 2.5 and 2.0 mM, respectively. Assays were therefore carried out in the absence (●) and presence (○) of EGTA, following the thiophosphorylation of myosin.

lowed us to assay the ATPase activity over a similar time period and determine the influence of Ca^{2+}. The results are shown in Figure 3 and clearly indicate that the presence or absence of Ca^{2+} had an insignificant effect on the actin-activated Mg^{2+}-ATPase activity of the phosphorylated myosin. The specific ATPase activity, at 37 °C, was 75 nmol of phosphate liberated min^{-1} (mg of myosin)$^{-1}$.

The presence of phosphatase in the chromatographically purified myosin would have been evident either by a progressive reduction in the ^{32}P content or by a decrease in ATPase activity, and hence a nonlinear ATPase vs. time curve. Since the phosphatase is thought not to be influenced by Ca^{2+}, both of these effects would be identical in the presence and absence of Ca^{2+}, and thus no change in the Ca^{2+} sensitivity of the ATPase activity would be detected. The presence of kinase as a contaminant, however, could lead to an apparent Ca^{2+} sensitivity if the phosphorylation level of the myosin is less than saturating. During the ATPase assay, the kinase would phosphorylate the vacant sites on the myosin and the ATPase activity would increase. Since the kinase is active only in the presence of Ca^{2+}, the ATPase activity in the absence of Ca^{2+} would not change, and a Ca^{2+}-sensitive ATPase response would be generated. This situation, in fact, was obtained in one of our initial experiments and a Ca^{2+}-sensitive ATPase response of approximately 50% was observed.

The addition of crude kinase (see Experimental Procedure) to the phosphorylated myosin resulted in the removal of ^{32}P from the myosin (Figure 2). This occurred at approximately equal rates in the presence or absence of Ca^{2+} and demonstrates that the phosphatase activity does not exhibit a marked Ca^{2+} dependence. In the presence of Ca^{2+}, the myosin light-chain kinase was active, and the loss of radioactivity represented a phosphatase-kinase catalyzed exchange of ^{32}P for ^{31}P. In the absence of Ca^{2+}, the loss of ^{32}P was due entirely to the action of the phosphatase, and an approximate phosphatase activity of 0.2 nmol of P_i/min can be calculated over the first 10 min of the reaction. This is less than 1% of the usual ATPase rate and indicates that the liberation of phosphate from the myosin light chains via the action of the phosphatase is unlikely to obscure the myosin-catalyzed hydrolysis of ATP; i.e., in this system the level of kinase-phosphatase linked phosphate production (pseudo-ATPase) is insignificant.

ATPγS Experiments. The object of these experiments was to establish whether or not the actin-activated ATPase activity of the thiophosphorylated myosin was dependent on Ca^{2+}, the assumption being that, since the thiophosphate group is thought to be resistant to the action of the phosphatase, it should be relatively simple to trap the myosin in a stable phosphorylated state. The experimental design was to preincubate gizzard myosin with varying concentrations of ATPγS and the myosin light-chain kinase in the presence of Ca^{2+} under the conditions used for ATPase assays. It was found that the presence or absence of actin and tropomyosin during this preincubation period did not affect the results, although actin was generally included as it was a requirement for the subsequent ATPase assays. Following the preincubation with ATPγS (usually 15 min at 25 °C), the ATPase reaction was started by the addition of ATP or ATP plus EGTA. The Ca^{2+} sensitivity of the actomyosin could therefore be measured and the influence of the pre-incubation with ATPγS determined.

Results from one experiment are shown in Figure 4. As the concentration of ATPγS was increased, the Mg^{2+}-ATPase activity in the absence of Ca^{2+} (presence of EGTA) increased until it was equal to the Mg^{2+}-ATPase activity measured in the presence of Ca^{2+}; that is, the Ca^{2+} sensitivity was lost. At low concentrations of ATPγS an activation of the Mg^{2+}-ATPase activity (plus Ca^{2+}) was usually observed. The extent of activation was variable but was never greater than 50% of the control level. The different degrees of activation were probably due to differences in the kinase-phosphatase balance. At low levels of phosphatase activity the equilibrium between the phosphorylated and dephosphorylated substrates would favor predominantly the phosphorylated form. With increasing levels of phosphatase activity the equilibrium would be shifted progressively toward the dephosphorylated form, and as a consequence of this the actin-activated ATPase activity would be reduced. However, in the presence of ATPγS the relative levels of phosphatase activity will not influence the equilibrium,

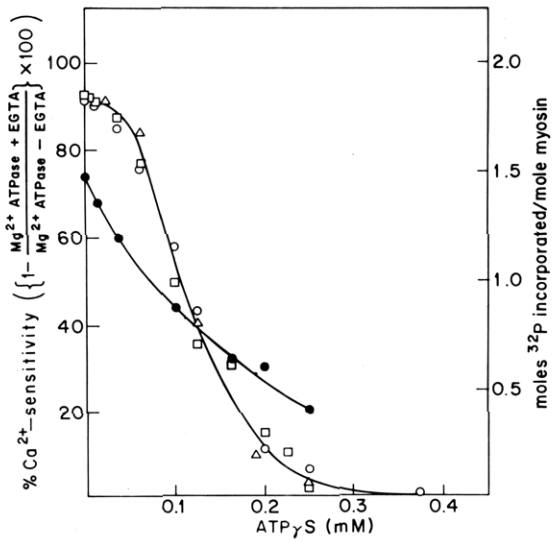

FIGURE 5: The effect of ATPγS on the Ca^{2+} sensitivity and ^{32}P incorporation with gizzard myosin. The solvent during the preincubation period with ATPγS was as given in Figure 4. Other conditions during the preincubation period for the ATPase assays were: gizzard myosin (1 mg/mL), skeletal actin (0.5 mg/mL), crude kinase (0.45 mg/mL). The ATPase assays were started after a 15-min preincubation by the addition of ATP or ATP plus EGTA. The final concentrations of ATP and EGTA were 2.5 and 2.0 mM, respectively. For the phosphorylation assays, skeletal actin was omitted; the assay was started after a 15-min preincubation with ATPγS by the addition of [γ-^{32}P]ATP (ca. 1 μCi of ^{32}P/assay) and the level of ^{32}P incorporation was determined after 10 min at 25 °C: ^{32}P incorporation (●); Ca^{2+} sensitivity for different preparations of gizzard myosin and kinase (□, ○, △).

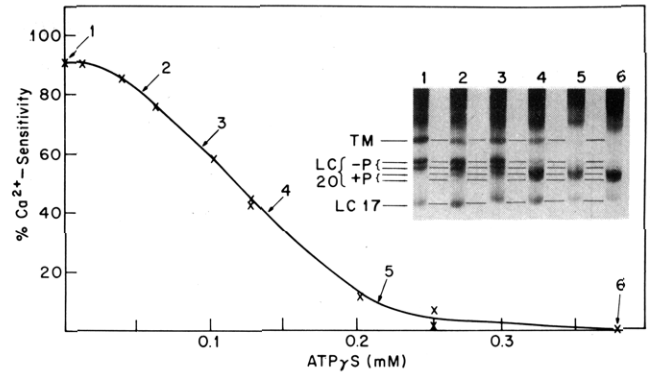

FIGURE 6: The effect of ATPγS on the 20 000-dalton light-chain population of gizzard myosin relative to Ca^{2+} sensitivity. The preincubation conditions with ATPγS and the determination of Ca^{2+} sensitivity were as given in Figure 4. Samples, as indicated, 1–6, for the urea–polyacrylamide gels were taken following the 15-min preincubation period with ATPγS, and solid urea was added to 7 M and dialyzed extensively against 20 mM Tris–glycine (pH 8.6), 7 M urea. Approximately 100-μg samples were applied to the urea–polyacrylamide gels for electrophoresis (Perrie and Perry, 1970). For samples 5 and 6, the preincubation with ATPγS was carried out using the kinase supernatant, 0.15 mg/mL (see Experimental Procedure) and, therefore, the tropomyosin band on the urea–polyacrylamide gel is not seen. For the determination of Ca^{2+} sensitivity on these samples, skeletal tropomyosin (0.1 mg/mL) was added. The abbreviations are: TM, tropomyosin; L.C. 20, the 20 000-dalton light chain; −P, +P, the unphosphorylated and phosphorylated light chains, respectively; L.C. 17, the 17 000-dalton light chain. The phosphorylated and unphosphorylated species of the 20 000-dalton light chains both appeared as a doublet.

since the thiophosphorylated substrate is resistant to hydrolysis by the phosphatase. The activation of Mg^{2+}-ATPase activity in the presence of ATPγS reflects the shift in the equilibrium position from the partially phosphorylated form (in the absence of ATPγS) to the saturated thiophosphorylated form and, therefore, is a measure of the phosphatase activity in that preparation. The results shown in Figure 4 are indicative of a "phosphatase poor" preparation. The varying responses of the Mg^{2+}-ATPase activity between different preparations can be normalized, however, by expressing the results on the basis of Ca^{2+} sensitivity. The results from three different preparations are given in Figure 5. The relationship between Ca^{2+} sensitivity and the concentration of ATPγS used in the preincubation period is reasonably consistent and shows that as the concentration of ATPγS is increased the control by Ca^{2+} is progressively lost.

These results indicate that the actin-activated ATPase activity of phosphorylated myosin is not affected by alterations in the Ca^{2+} concentration. However, before this can be accepted it is necessary to demonstrate that the sites of phosphorylation are identical using either ATP or ATPγS as substrates. One approach to this problem was to measure the degree of ^{32}P incorporation following exposure to different concentrations of ATPγS. If identical sites are involved, then preincubation with ATPγS should block the subsequent incorporation of ^{32}P from [γ-^{32}P]ATP. This was indeed found to be the situation, as shown in Figure 5. The incorporation of phosphate was approximately 1.5 mol of P/mol of myosin for the control sample, and this was reduced to about 0.4 mol of P/mol of myosin following preincubation with 0.25 mM ATPγS.

An alternate approach was to examine the population of phosphorylated 20 000-dalton light chains following exposure to different concentrations of ATPγS. To do this, urea–polyacrylamide gel electrophoresis was used (see Experimental Procedure), as it was shown previously that this system could distinguish between the phosphorylated and nonphosphorylated light chains of skeletal myosin (Perrie and Perry, 1970). The results are given in Figure 6. As the concentration of ATPγS was increased, the proportion of the thiophosphorylated light chain increased, and this in turn was related to the degree of Ca^{2+} sensitivity, the higher levels of thiophosphorylated light chain being associated with a reduced Ca^{2+} sensitivity. For example, a sample taken as indicated at position 3 showed both the thiophosphorylated and unphosphorylated light chains and had an intermediate level of Ca^{2+} sensitivity. In the next sample, the Ca^{2+} sensitivity was reduced and this was accompanied by a decrease in the unphosphorylated light chain. Finally, at position 6, where the Ca^{2+} sensitivity was abolished, only the thiophosphorylated light chain was visible. [The position of the phosphorylated 20 000-dalton light chain of gizzard myosin, under our gel conditions, was established using [γ-^{32}P]ATP as described earlier by Górecka et al. (1976)].

From the above experiment it is reasonable to conclude that the same sites were labeled using either ATP or ATPγS, as the substrate for the myosin light-chain kinase and that the myosin frozen in the thiophosphorylated state was not inhibited in the absence of Ca^{2+}.

Several controls were performed and, since these were critical for the validity of the above suggestion, they will be mentioned briefly. Preincubation with ATPγS of the following mixtures did not perturb the normal response: crude kinase, myosin plus or minus actin (i.e., in the absence of kinase), myosin plus kinase plus EGTA. The possibility of a chemical modification of the myosin light chains by ATPγS (presumably via a disulfide bond) was ruled out by the inclusion of dithiothreitol, at a final concentration of 5 mM, during the ATPγS incubation period.

The Correlation of Dephosphorylation with Inactivation of ATPase Activity. The type of experiment that was carried

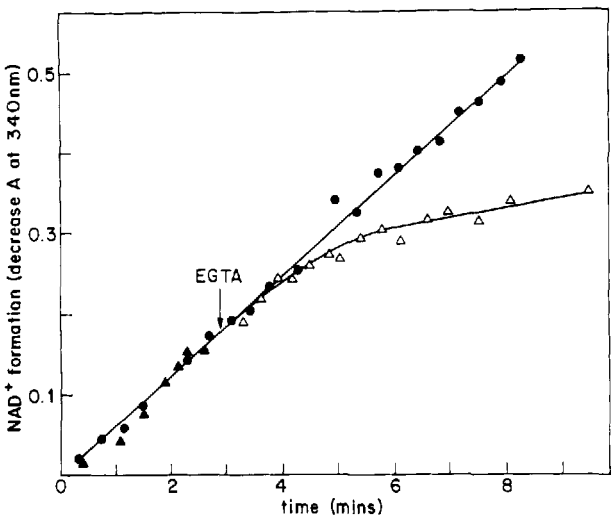

FIGURE 7: The Mg^{2+}-ATPase activity following the removal of Ca^{2+} using a system containing gizzard myosin. ATPase conditions: 50 mM KCl, 11.5 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris-HCl (pH 7.5), gizzard myosin (2.0 mg/mL), skeletal actin (0.26 mg/mL), skeletal tropomyosin (0.13 mg/mL), kinase supernatant (0.1 mg/mL), 2.85 mM phosphoenolpyruvate, pyruvate kinase (20 units/mL), lactate dehydrogenase (29 units/mL), 0.9 mM NADH. Two assays were performed. One assay served as the $+Ca^{2+}$ control. To the other assay, EGTA (final concentration 2 mM) was added at the point indicated, the sample was rapidly mixed, and the formation of NAD^+ was monitored using the aliquot-sodium dodecyl sulfate method (see Experimental Procedure).

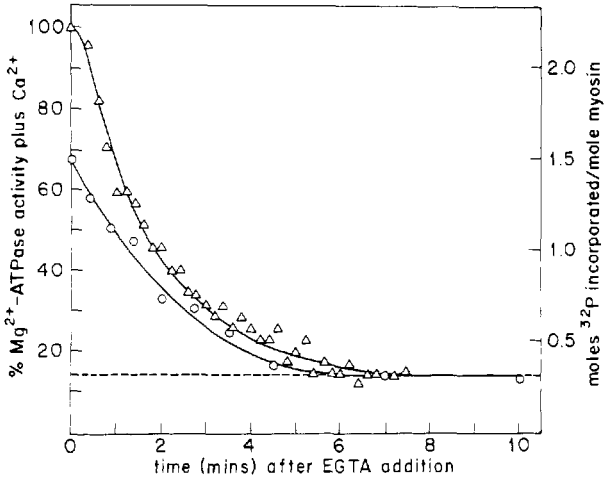

FIGURE 8: Correlation of the rate of actin-activated Mg^{2+}-ATPase activity and the dephosphorylation of gizzard myosin following the removal of Ca^{2+}. The conditions and procedure for the ATPase assays were as given in Figure 7. The Mg^{2+}-ATPase activity in the presence of Ca^{2+} was established (approximately 10 min after the addition of ATP), EGTA was added (zero time on the abscissa), and aliquots were withdrawn for the determination of NAD^+. For the determination of ^{32}P content, a similar procedure was followed, except [γ-^{32}P]ATP was used, approximately 1 μCi/aliquot: ATPase activity (△), ^{32}P incorporation (○).

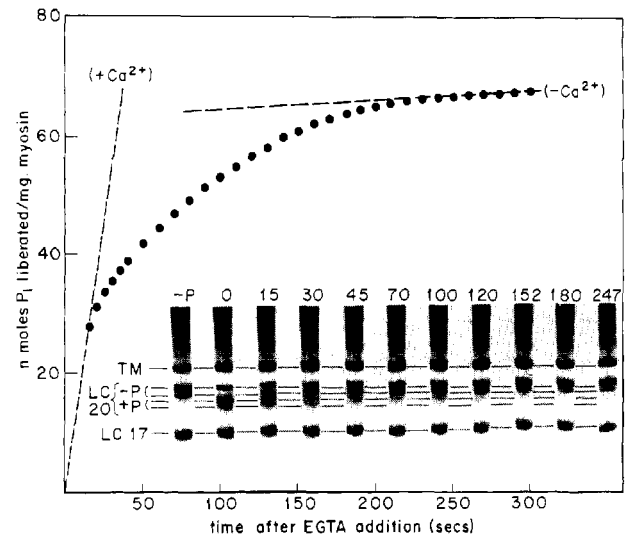

FIGURE 9: The population of the 20 000-dalton light chain of gizzard myosin following the removal of Ca^{2+}. The conditions for the ATPase assays were as given in Figure 7, except that the continuous monitoring system using an Aminco DW-2 spectrophotometer (see Experimental Procedure) was used. The actin-activated Mg^{2+}-ATPase activity in the presence of Ca^{2+} was established (dashed line, $+Ca^{2+}$), EGTA was added (zero time on the abscissa), and NAD^+ formation was recorded. Samples were also withdrawn at the times indicated for urea-polyacrylamide gel electrophoresis. The procedure and the abbreviations used are given in Figure 6.

out is as follows: The steady-state rate of Mg^{2+}-ATPase activity in the presence of Ca^{2+} was established for various actomyosin mixtures, EGTA was then added, and the progressive decrease in ATPase activity and myosin phosphorylation was followed until the stable $-Ca^{2+}$ levels were obtained. It was reasoned that the removal of Ca^{2+} from protein sites by EGTA would be relatively rapid, whereas the removal of phosphate from the myosin light chains might take longer. Thus, the rate at which the actomyosin was "turned off" should follow one of these two processes. As a test of this hypothesis, we used a system which is known to be regulated via a Ca^{2+}-protein interaction, i.e., skeletal subfragment I, actin, troponin, and tropomyosin. Following the addition of EGTA, the inhibited rate of ATPase activity was established within the time required for mixing the sample, i.e., in less than 15 s. This is obviously much too long to be a reasonable estimate of the relaxation rate in skeletal muscle, as this should occur on a millisecond time scale. However, the experiment did serve to illustrate the general point that if relaxation depends predominantly on the removal of Ca^{2+} from a regulatory site then this process would be too fast to detect using this technique.

In a similar experiment using gizzard myosin, skeletal actin, and crude kinase (see Experimental Procedure), a marked lag period occurred after the addition of EGTA and before the linear $-Ca^{2+}$-ATPase rate was established (Figure 7). Thus, in this system the time taken to achieve relaxation (approximately 3 min) was considerably longer than in the previous example using skeletal muscle proteins. It is unlikely that Ca^{2+} removal by EGTA would require this prolonged time period, and the results suggest that the dephosphorylation rate might be the dominant time determinant in relaxation. To test this suggestion, the ATPase activity following the addition of EGTA was measured and correlated over the same time period to the degree of myosin phosphorylation. The results are presented in Figure 8. As before, the gradual decrease in ATPase activity occurred over a relatively long time period and did not attain the stable relaxed (minus Ca^{2+}) rate until approximately 7 min after the addition of EGTA. The critical point, however, is that the dephosphorylation of the myosin light chains occurred over the same period and followed similar kinetics, thus strengthening the theory that the relaxation process is governed by the phosphatase rate.

From the above results it is evident that the ATPase activity is proportional to the level of myosin phosphorylation, and to investigate this relationship further we analyzed the light-chain populations at different times following EGTA addition, using the urea-polyacrylamide gel electrophoresis system. The results are shown in Figure 9. The myosin as it is isolated from

gizzards exists in the dephosphorylated form (−P in Figure 9). The zero time point shows the extent of phosphorylation that occurred during the ATPase assay in the presence of Ca^{2+}. Both the phosphorylated and dephosphorylated forms of the 20 000-dalton light chains were present, although the phosphorylated light chain was the major species. Following the addition of EGTA, the proportion of the phosphorylated light chain was progressively reduced until after approximately 180 s it was not detected on the urea–acrylamide gels.

All of the results that have been presented above are consistent with the hypothesis that the Mg^{2+}-ATPase activity of gizzard myosin plus skeletal actin is dependent upon the state of phosphorylation of the myosin molecule and is not dependent upon the occupancy by Ca^{2+} of specific sites on the myosin molecule.

Discussion

The observation that phosphorylation of the myosin molecule is a prerequisite for activation of ATPase activity by actin has been proposed before (Sobieszek, 1977a,b; Górecka et al., 1976; Chacko et al., 1977), and a close correlation of these two events is also evident from the above results. The situation that we wished to clarify, however, was concerned with the role of Ca^{2+} in the relaxation process of smooth muscle. Each of the three experimental approaches that were described indicated that the dominant role of Ca^{2+} is to control the activity of the myosin light-chain kinase and the presence of other critical Ca^{2+} sites, presumably on the myosin molecule, is not consistent with the data. In other words, if the myosin molecule is phosphorylated, then its actin-activated ATPase activity is not influenced by the concentration of Ca^{2+}. This agrees with the findings of Small and Sobieszek (1977a,b) using preparations from pig stomach and chicken gizzard, respectively, but is at variance with those of Chacko et al. (1977), who used myosin isolated from guinea pig vas deferens. It is possible of course that myosin isolated from various sources is subject to control by different mechanisms; for example, this is accepted in the case of skeletal and molluscan myosins. However, in this discussion our concern is only with those systems that are thought to be activated via phosphorylation of the myosin molecule and it is relevant to ask whether or not it is reasonable to expect a diversity of regulatory mechanisms within this category. Several examples of a Ca^{2+}-independent myosin light-chain kinase have been cited (Daniel and Adelstein, 1976; Adelstein et al., 1976, 1977). The situation therefore exists where myosin can be phosphorylated in the absence of Ca^{2+}, and the Ca^{2+} dependence of the actin-activated ATPase activity of phosphorylated myosin can be tested. If the ATPase activity is Ca^{2+} insensitive, then this would indicate that the particular myosin does not possess a Ca^{2+}-binding "on–off" switch of the molluscan type. Examples include platelet myosin (Adelstein et al., 1976) and myoblast myosin (Scordilis and Adelstein, 1977). Also, it has been shown that a Ca^{2+}-insensitive kinase is produced following limited proteolysis of the Ca^{2+}-dependent gizzard kinase (Hartshorne et al., 1977b). The actin-activated ATPase activity of gizzard myosin phosphorylated by this altered kinase is not inhibited on the removal of Ca^{2+}. In other experiments it was shown recently that functionally skinned fibers of rabbit ileum (Cassidy et al., 1978), chicken gizzard, and rabbit pulmonary artery (Kerrick, Hoar, and Cassidy, personal communication) lost Ca^{2+} sensitivity but were fully activated following incubation with ATPγS. Thus, the myosin from vas deferens (Chacko et al., 1977) is unique, as it is the only myosin within this category that requires Ca^{2+} bound to myosin in addition to the phosphorylation of the light chains for full actin-activated ATPase activity. Obviously, this possibility cannot be excluded, but, in our opinion, it is unlikely and should be reexamined in future studies.

One of the experimental approaches that was used in the above studies was to correlate the phosphatase rate with the rate of inhibition of Mg^{2+}-ATPase activity after the removal of Ca^{2+}. The time needed to establish a linear inhibited ATPase activity was variable but occurred over a period of minutes. This is probably too slow to be directly applicable to the intact muscle. Our studies were intended not to define the physiological relaxation rate but to distinguish between the rates of Ca^{2+} and phosphate removal and to decide which of these correlated with the in vitro relaxation rate. Clearly, a low phosphatase activity facilitated the choice. The phosphatase rates also allowed some estimates to be made of the kinase–phosphatase-linked phosphate release ("pseudo-ATPase"). These were estimated for several preparations of actomyosin and rarely exceeded 1 nmol of P_i liberated min^{-1} (mg of actomyosin)$^{-1}$. Thus, although these levels were probably lower than those in the intact muscle, the contribution of pseudo-ATPase activity to the overall P_i release, in the in vitro studies, was negligible. This is significant not only from a practical point of view but it contributes also to our knowledge of the proposed mechanism for ATP hydrolysis (see Figure 1); i.e., since the pseudo-ATPase rate is so low, each phosphorylated myosin molecule must catalyze the hydrolysis of many ATP molecules before it is dephosphorylated. If dephosphorylation and rephosphorylation of the 20 000-dalton light chain were required for each successive molecule of ATP hydrolyzed by myosin (considering only one hydrolytic site), then the pseudo-ATPase activity would equal the actin-activated Mg^{2+}-ATPase activity of myosin. This is not the case, and therefore each molecule of phosphate incorporated into the myosin light chain must be retained through several cycles of ATP hydrolysis. It is assumed that for each one or two molecules of ATP hydrolyzed there will be an accompanying association and dissociation of the actomyosin complex. (In the in situ muscle, this would correspond to the repetitive cross-bridge cycling which is responsible for contraction.) It follows then that dephosphorylation of myosin is not a prerequisite for the dissociation of the actomyosin complex. Our contention, however, is that dephosphorylation of myosin is a prerequisite for relaxation and that the removal of Ca^{2+} from sites on myosin is not capable of overriding the influence of phosphorylation. The muscle remains in the dissociated, or relaxed, state as long as the myosin is not phosphorylated. This statement applies to the fully relaxed state and does not include the intermediate rigorlike state observed with several smooth muscles (Siegman et al., 1976). The latter is thought to be due to noncycling cross-bridges which are attached at intermediate Ca^{2+} levels (i.e., below the activating level). On the basis of our experiments, we would expect that the myosin in this rigorlike condition is not phosphorylated. Thus, although it is entirely speculative, it might be suggested that the attachment and detachment of these inactive cross-bridges could be regulated by the binding of Ca^{2+} to myosin.

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