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Regulation of the Actin–Myosin Interaction in Vertebrate Smooth Muscle: Activation *via* a Myosin Light-chain Kinase and the Effect of Tropomyosin

APOLINEY SOBIESZEK† AND J. V. SMALL‡

*Department of Molecular Biology, Aarhus University
8000 Aarhus C, Denmark*

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The precipitation of smooth muscle actomyosin by ammonium sulphate at 25% saturation results in the removal of both tropomyosin and a myosin light-chain kinase and in the concomitant and complete loss of actin-activated ATPase activity. Normal activity and Ca-sensitivity may then be readily restored by the recombination of the tropomyosin-kinase fraction with the precipitated actomyosin. The same tropomyosin-kinase fraction was found to produce a twofold increase in the activity of reconstituted myosin and actin from smooth muscle to a level characteristic for natural actomyosin and myosin plus thin filaments. Experiments in which the added amount of tropomyosin-kinase fraction was gradually increased showed that the increase in actin-activated ATPase activity was associated with a parallel increase in the rate of phosphorylation of the myosin light chain.

A similar activation of ATPase activity by the tropomyosin-kinase fraction was noted with myosin and actin prepared from skeletal muscle, but, in contrast to the situation with smooth muscle, this activation was Ca-insensitive. Furthermore, from the absence of any phosphorylation of the skeletal muscle light chains the activation in this system could be attributed to the presence of gizzard tropomyosin alone.

The myosin light-chain kinase was found to be enriched in extracts obtained as by-products during the preparation of natural actomyosin and could be extracted directly from homogenized and washed smooth muscle in the absence of ATP. Experiments with such kinase extracts as well as with partially purified preparations of the light-chain kinase obtained by gel filtration showed that both the kinase and Ca were obligatory for the stimulation of actin-activated ATPase activity. But full activity, characteristic of natural actomyosin was only obtained in the presence of tropomyosin which produced a two to threefold stimulation of the actin-activated ATPase activity above that obtained with the light-chain kinase alone.

1. Introduction

Earlier studies (Bremel, 1974; Mrwa & Rüegg, 1975; Sobieszek & Bremel, 1975; Sobieszek & Small, 1976) have shown that the Ca^{2+} regulation of vertebrate smooth muscle is, as in certain invertebrate muscles (Kendrick-Jones *et al.*, 1970; and see reviews by Szent-Györgyi, 1975; Lehman, 1976) associated with the myosin molecule.

† Present address: Section on Molecular Cardiology, Cardiology Branch, National Heart, Lung and Blood Institute, Bethesda, Md 20014, U.S.A.

‡ Present address: Institute of Molecular Biology of the Austrian Academy of Sciences, 5020 Salzburg, Austria.

More recently, evidence has been obtained which indicates that the triggering by Ca of the actin-myosin interaction in smooth muscle is effected *via* a Ca-dependent phosphorylation of the 20,000 molecular weight light chain of myosin and that this, in turn is dependent on an endogenous light-chain kinase (Sobieszek, 1977a,b). The presence of such a kinase in smooth muscle has also been noted by other investigators (Frearson *et al.*, 1976; Aksoy *et al.*, 1976).

In recombination experiments with smooth muscle preparations (Sobieszek & Small, 1976) it was found further that the actin-activated ATPase activity of myosin and F-actin was about half that obtained with myosin and thin filaments, composed of only actin and tropomyosin, and it was thus suggested that tropomyosin may serve some "amplifying" function in this actomyosin system. A Ca-independent stimulation of actin-activated ATPase activity by tropomyosin has been noted in *Limulus* actomyosin (Lehman & Szent-Györgyi, 1972) and in skeletal muscle actomyosin at low MgATP concentrations (Bremel & Weber, 1972; Bremel *et al.*, 1973; Shigekawa & Tonomura, 1972, 1973) and it was considered likely that these phenomena were related to that observed in smooth muscle. However, since the presence of different amounts of myosin light-chain kinase in the F-actin and thin filament preparations used in the earlier experiments could not be excluded it was necessary to investigate further the relative contributions of the light-chain kinase and tropomyosin to the activation process.

Using preparations obtained from smooth muscle actomyosin which contain the light-chain kinase alone or together with tropomyosin, as well as conventionally prepared tropomyosin, we have been able to investigate the separate effects of these components. In these studies as described in this paper, we have utilised, in addition to reconstituted actomyosins, actomyosin from which both tropomyosin and the light-chain kinase have been completely removed. The effects of tropomyosin and the light-chain kinase from smooth muscle have also been tested on reconstituted actin and myosin from skeletal muscle. The results show that while the myosin light-chain kinase is, as expected, obligatory for the activation of the actomyosin interaction, tropomyosin serves to increase further the actin-activated ATPase activity, presumably by effecting a "co-operative interaction" between actin monomers on the thin filament.

2. Materials and Methods

(a) Preparation of actomyosin

Smooth muscle actomyosin was prepared from fresh chicken or turkey gizzard as described previously (Sobieszek & Bremel, 1975; Sobieszek & Small, 1976). Briefly, crude actomyosin was extracted from a myofibril-like preparation at low ionic strength in the presence of ATP, and purified by precipitation by the addition of 25 mM-MgCl₂ or 15 mM-CaCl₂. Accordingly, we term the resulting actomyosins MgAM and CaAM, respectively. In our earlier studies (Sobieszek & Small, 1976) we observed that low concentrations of CaCl₂ (1 to 2 mM), while being effective in precipitation, resulted in an actomyosin with very variable Ca-sensitivity. In contrast, full Ca-sensitivity, as exhibited by MgAM was later found to be retained by using the 15 mM-CaCl₂.

Tropomyosin and the light-chain kinase were removed from Ca- or Mg-actomyosin by precipitation with ammonium sulphate at 25% saturation. In the case of MgAM, for which the ammonium sulphate pellet was less compact than with CaAM, 2 precipitations at 25% saturated ammonium sulphate were necessary to ensure the complete removal of tropomyosin. The actomyosins were subsequently pelleted and washed twice in a "wash

solution" (60 mM-KCl, 1 mM-MgCl₂, 20 mM-imidazole, 1 mM-cysteine, pH 6.8 at 4°C) to remove ammonium sulphate.

(b) *The tropomyosin and myosin light-chain kinase fraction*

The supernatant fraction obtained by ammonium sulphate precipitation of actomyosin contained, together with tropomyosin, relatively large amounts of the myosin light-chain kinase. These were co-precipitated at 55% ammonium sulphate saturation and pelleted by centrifugation at 15,000 g for 30 min. The pellet was then dissolved in and dialysed against wash solution to remove ammonium sulphate and subsequently against a solution used as a stock solution for the ATPase assays (60 mM-KCl, 40 mM-imidazole, pH 7.0 at 25°C and freshly added 1 mM-cysteine). After dialysis the insoluble proteins were removed by centrifugation at 45,000 g for 30 min. The resulting fraction contained small amounts of a 220,000 molecular weight protein (Sobieszek & Bremel, 1975) present in the original actomyosin and which could be removed by an intermediate precipitation at 40% ammonium sulphate saturation. The fractions obtained between 25% and 55%, or 40% and 55%, ammonium sulphate saturation both contained tropomyosin and the myosin light-chain kinase and we refer to these as the "tropomyosin-kinase fractions".

(c) *Myosin light-chain kinase fraction and purification*

The supernatant fraction obtained by the precipitation of actomyosin with MgCl₂ was found to be rich in the myosin light-chain kinase and the 220,000 molecular weight protein and contained very low amounts of tropomyosin. After raising the pH to 7.6 to 8.0 to precipitate some of the residual tropomyosin, this supernatant was cleared by centrifugation and then dialysed against the ATPase assay stock solution to remove inorganic phosphate. The insoluble components were finally removed by centrifugation as described above. While tropomyosin could be totally removed from the light-chain kinase fraction by isoelectric precipitation at pH 4.6 this resulted in the complete loss of kinase activity.

The light-chain kinase was also obtained by direct extraction of myofibrils in the absence of ATP. Extraction of the kinase was found to be facilitated at higher ionic strength ($\mu = 0.12$) and at alkaline pH (7.4 to 7.8) with the inclusion of MgCl₂ to minimize extraction of actin and tropomyosin. The composition of the extraction medium was the same as for the ATPase assay stock solution but with the pH adjusted to 7.4 to 7.8 (at 4°C) and with added 15 to 30 mM-MgCl₂. The extract was dialysed against ATPase assay stock solution to precipitate insoluble material and the kinase purified by gel filtration in the same buffer on Sepharose 6B or Sephadex G150.

(d) *Recombination experiments*

Individual contractile proteins were purified from chicken and turkey gizzard and rabbit skeletal muscle as described previously (Sobieszek & Bremel, 1975; Sobieszek & Small, 1976).

Recombination experiments were carried out in one of two ways. In the first type of experiment the assay tubes contained progressively greater proportions of actin to myosin and, where appropriate, added amounts of the tropomyosin-kinase fraction to give a constant actin to tropomyosin ratio. Compensation for the increase in viscosity at high actin to myosin ratios was achieved by progressively reducing the amount of added myosin.

In the second type of experiment, actomyosin (MgAM 25 or CaAM 25) or recombined myosin and actin mixed together in a constant ratio were introduced in a fixed amount to the assay tubes. Additional tropomyosin, the tropomyosin-kinase or the kinase fraction, were then added to the tubes in progressively increasing amounts. Where gizzard tropomyosin prepared by the Bailey (1948) procedure was added together with the kinase fraction, lyophilised tropomyosin was dissolved directly in the kinase fraction at a concn of 5 mg/ml before addition of the fraction to the assay tubes.

In both sets of experiments the assay volume (2.5 ml before the addition of ATP) was made up by the addition of appropriate amounts of ATPase assay stock solution.

(e) *ATPase activity and phosphorylation assays*

Measurements of ATPase activity were carried out at 25°C as described previously (Sobieszek & Small, 1976) using a final assay volume of 3 ml and in the presence of 2 mM-MgCl₂ and either 0·1 mM-CaCl₂ or 2 mM-EGTA. The reaction was initiated by the addition of 0·5 ml of 6 mM-ATP dissolved in the ATPase assay stock solution and terminated, after 30, 45 or 60 s, by the addition of 2 ml of 10% trichloroacetic acid. The contents of each assay tube were then filtered and the inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

For the measurement of ATPase activity at different ATP concentrations, phosphate liberation was determined from the amount of creatine liberated in the presence of an ATP-regenerating system (c.f. Weber, 1969) containing 0·5 mg creatine kinase/ml and 5 mM-creatine phosphate. The conditions were otherwise the same as those used for the normal ATPase assays but with a total assay volume of 1·5 ml. The reaction was initiated by the addition of 0·25 ml of a solution containing ATP together with the creatine phosphate and was terminated by the addition of *p*-hydroxymercuribenzoate. Liberated creatine was estimated according to the Sigma bulletin no. 520.

Myosin light-chain kinase activity of different preparations was determined from the incorporation of radioactive phosphorus into a fixed amount of purified 20,000 molecular weight smooth muscle myosin (L₂₀) light chain, as described elsewhere (Perrie & Perry, 1970; Sobieszek, 1977b). For fractions rich in kinase activity it was found that the saturation for phosphate incorporation occurred, for undiluted preparations, within the first 1 to 3 s. The measurement of phosphorylation rate was, in such cases, carried out with the preparations diluted 3 to 5 times, and with assay times of 3 to 30 s. Less active preparations were assayed undiluted using the same incubation times.

(f) *Dodecyl sulphate/gel electrophoresis and protein determination*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969) with some modifications (Sobieszek & Bremel, 1975).

Protein concentrations were determined by the Biuret method using a protein standard from Sigma Corp. for calibration.

3. Results

(a) *Activating effect of the tropomyosin-myosin light-chain kinase fraction on tropomyosin-free actomyosin*

It was previously shown (Sobieszek & Bremel, 1975) that tropomyosin may be effectively removed from smooth muscle actomyosin by precipitation with ammonium sulphate at 25% saturation. For actomyosins prepared, in the present study, by the use of MgCl₂ or CaCl₂ precipitation, this fractionation was also found to result in tropomyosin-free actomyosins (Figs 1(c) and 2(a)) and which we shall refer to as MgAM 25 and CaAM 25, respectively. Such actomyosins consistently exhibited no actin-activated ATPase activity. When, however, the tropomyosin-kinase fraction obtained between 25% and 55% (Fig. 2(f)) or 40% and 55% (Fig. 2(g)) ammonium sulphate saturation (see Materials and Methods) was recombined with these actomyosins they then exhibited actin-activated ATPase activity and Ca-sensitivity characteristic of the parent actomyosin. The activating effect of the tropomyosin-kinase fraction is shown in Figure 3. For MgAM 25 the activation was somewhat less than for CaAM 25 but the optimal activity was still of the order of that obtained with natural actomyosin. A saturation of the effect occurred over a fairly broad range but in the region of about 300 µg of tropomyosin per mg of actomyosin.

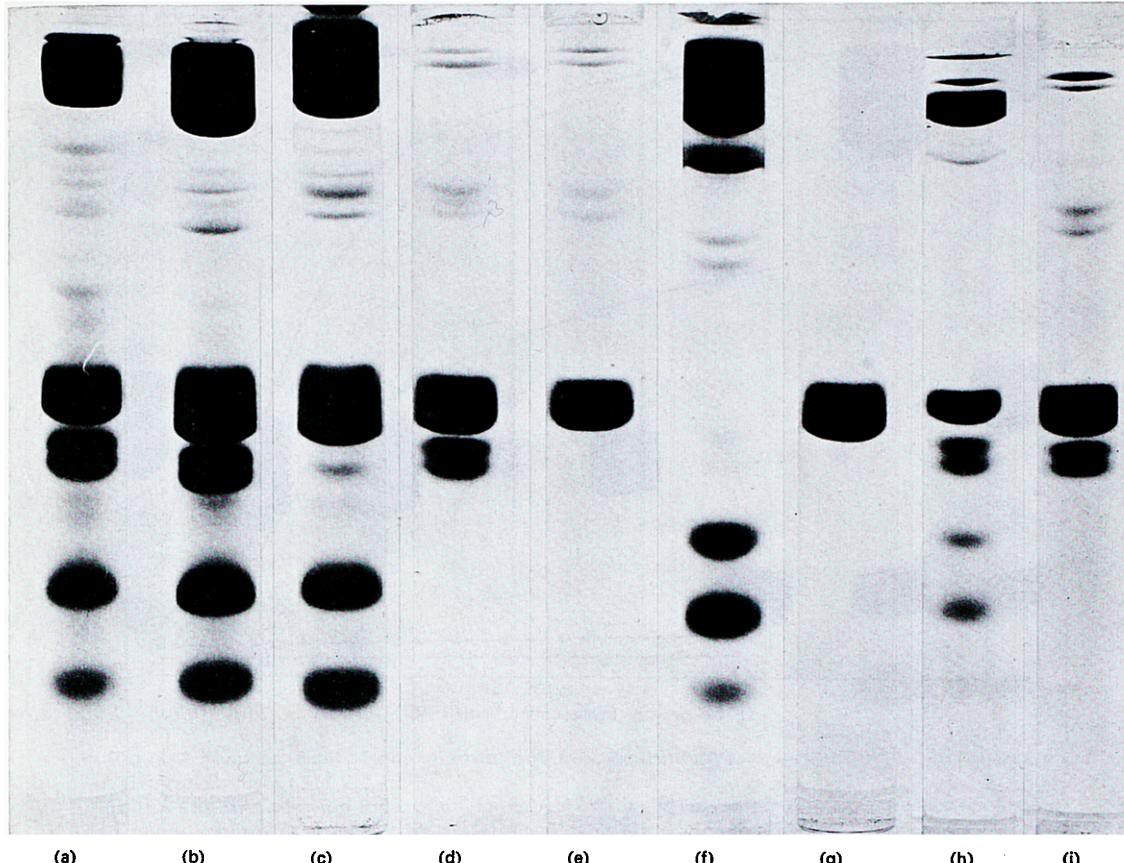


FIG. 1. Sodium dodecyl sulphate/polyacrylamide gels of the following preparations:

(a) Crude actomyosin extracted from chicken gizzard myofibrils. Major bands are, from top to bottom, the myosin heavy chain, actin, tropomyosin, and the 20,000 molecular weight (L_{20}) and 17,000 molecular weight (L_{17}) myosin light chains.

(b) Purified gizzard actomyosin (MgAM).

(c) Tropomyosin-free gizzard actomyosin (CaAM 25) obtained after one precipitation with ammonium sulphate at 25% saturation.

(d) and (e) Chicken gizzard thin filaments and F-actin, respectively. The thin filaments were extracted from myofibrils using an extraction medium containing 3 mM-MgCl₂, 2 mM-ATP, 60 mM-KCl, 1 mM-cysteine, 40 mM-imidazole (pH 6.5) and were collected by centrifugation at 40,000 revs/min for 2 h. F-actin was obtained by the sedimentation of the thin filament extract with the KCl concn raised to 0.5 M and the pH to 7.0.

(f) and (g) Skeletal muscle myosin and F-actin, respectively.

(h) and (i) Binding of tropomyosin in the tropomyosin-kinase fraction to actomyosin from rabbit skeletal muscle (h) and chicken gizzard F-actin (i). For (h), reconstituted skeletal muscle actomyosin was mixed with the tropomyosin-kinase fraction and the pellets obtained by centrifugation washed twice to remove unbound tropomyosin. For (i), gizzard F-actin was mixed with the tropomyosin-kinase fraction and the thin filament complexes collected by high-speed centrifugation.

No difference was observed between the activating effects of the two fractions obtained from the two different fractionations of the 25% ammonium sulphate supernatant. This indicated that the 220,000 molecular weight protein present in the 25 to 55% tropomyosin-kinase fraction (see Fig. 2(f)) was not an essential component of Ca-sensitive actomyosin.

Parallel measurements of the rate of phosphorylation of tropomyosin-free acto-

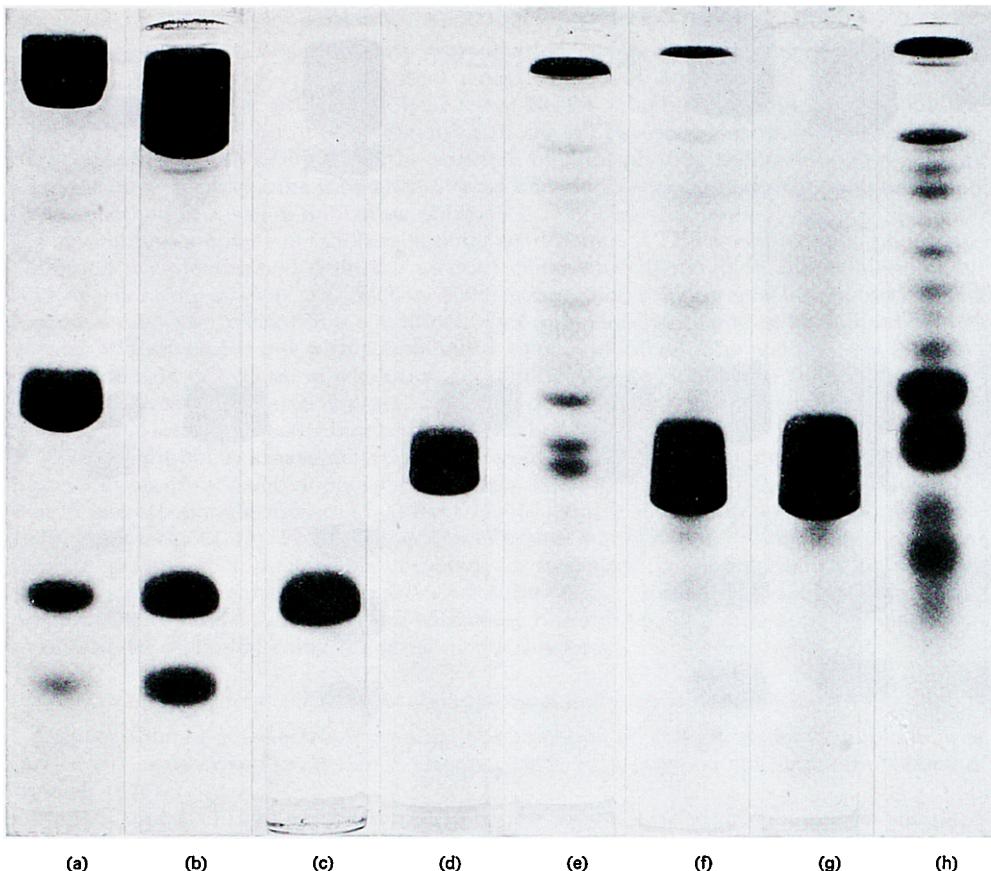


FIG. 2. Sodium dodecyl sulphate/polyacrylamide gels of further chicken preparations.

- (a) Tropomyosin-free gizzard actomyosin (MgAM 25 II) obtained after 2 ammonium sulphate precipitations.
- (b) Chicken gizzard myosin.
- (c) Chicken gizzard L_{20} myosin light chain purified by guanidine chloride/ethanol fractionation of myosin.
- (d) Tropomyosin from chicken gizzard prepared according to the Bailey (1948) procedure. This tropomyosin typically runs as a doublet.
- (e) Myosin light-chain kinase-rich fraction obtained as the supernatant after Mg-precipitation of crude actomyosin. The upper band in this preparation, as well as in (f) corresponds to the 220,000 molecular weight protein (see text) and not to myosin.
- (f) and (g) Fractions rich in tropomyosin and the myosin light-chain kinase (tropomyosin-kinase fractions) obtained by ammonium sulphate fractionation of purified actomyosin in the range of 25 to 55% and 40 to 55% saturation, respectively. At the very high protein loadings used for these gels the tropomyosin doublet is not seen.
- (h) Light-chain kinase extract of gizzard myofibrils.

myosin with increasing amounts of the tropomyosin-kinase fraction showed that the rate of phosphate incorporation into the light chain followed closely the measured actin-activated ATPase activity (Fig. 3). As shown elsewhere (Sobieszek, 1977*a,b*) the myosin phosphorylation was completely Ca-dependent.

Measurements showed that the tropomyosin-kinase fraction alone exhibited no ATPase activity. In addition, the absence of any phosphate liberation when this fraction was mixed with either smooth muscle myosin or the L_{20} myosin light chain demonstrated the absence of any contaminating specific light-chain phosphatase.

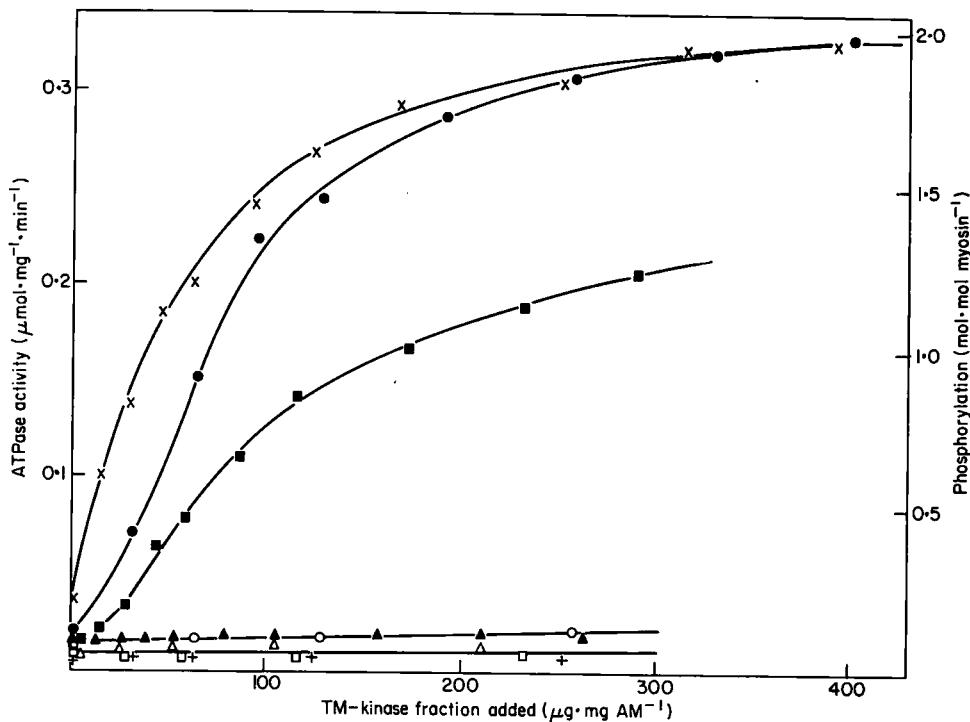


FIG. 3. Activating effect of tropomyosin (TM) and the tropomyosin-kinase fraction on tropomyosin-free actomyosin (AM 25).

In this and the following Figures solid symbols (●, ▲, ■) correspond to the presence of calcium and open symbols (○, △, □) to the absence of calcium. Squares (□, ■) and circles (○, ●) correspond, respectively, to the actin-activated ATPase activities of MgAM 25 and CaAM 25 with the addition of increasing amounts of the tropomyosin-kinase fraction. The corresponding rate of phosphorylation of the L₂₀ myosin light chain for CaAM 25 with added tropomyosin-kinase fraction is shown by the curves marked with crosses (—x—x—, calcium present; —+—+—, calcium absent). Tropomyosin alone had no activating effect (△, ▲).

(b) Activating effect of tropomyosin-kinase fraction on reconstituted actomyosin

Figure 4(a) shows the effect of the tropomyosin-kinase fraction on the ATPase activity of actomyosin reconstituted from gizzard myosin and gizzard F-actin for increasing ratios of actin to myosin. Where the tropomyosin-kinase fraction was added (squares) the mass ratio of tropomyosin to actin was maintained constant at a value of 0.3. Under these conditions there was an activation of the ATPase activity to a level of more than twice that observed with gizzard myosin and gizzard F-actin alone (triangles). This level of activation also corresponded to that observed with gizzard thin filaments (circles). The maximal activity obtained with these reconstituted systems was between 0.2 and 0.3 $\mu\text{mol mg myosin}^{-1} \text{ min}^{-1}$, that is about the same as obtained with natural actomyosin (Sobieszek & Small, 1976).

With actomyosin reconstituted from gizzard myosin and skeletal muscle F-actin the tropomyosin-kinase fraction produced an approximately fourfold activation of the ATPase activity (Fig. 4(b)). The maximal activity was, however, lower ($\sim 0.14 \mu\text{mol mg myosin}^{-1} \text{ min}^{-1}$) than that observed in the corresponding experiment with gizzard F-actin.

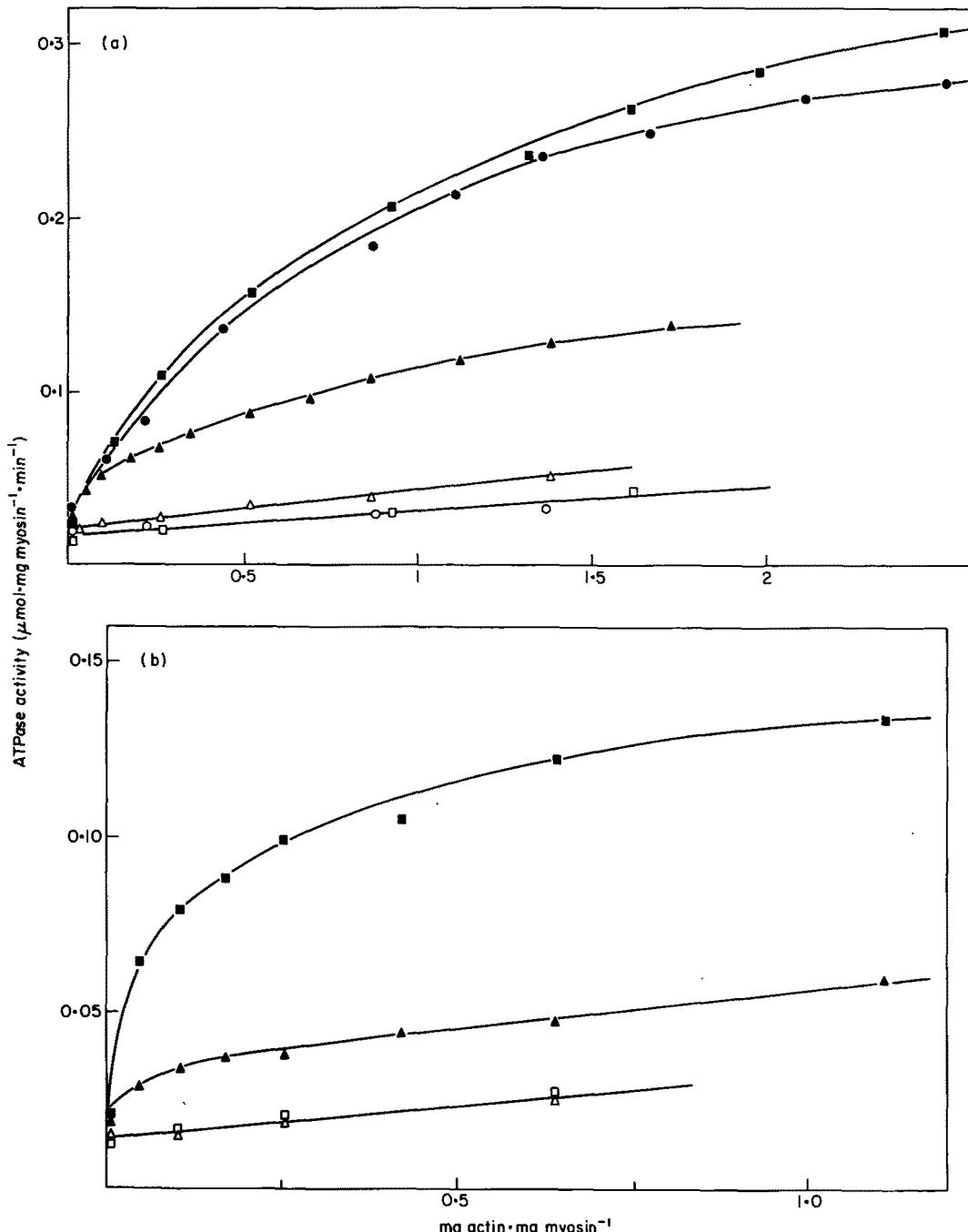


FIG. 4. (a) Actin-activated ATPase activities of reconstituted gizzard actomyosins for increasing ratios of actin to myosin. (Δ , \blacktriangle) Gizzard myosin plus gizzard F-actin; (\circ , \bullet) gizzard myosin plus gizzard thin filaments; (\square , \blacksquare) gizzard myosin plus gizzard F-actin together with added amounts of the tropomyosin-kinase fraction to give a fixed weight ratio of actin to tropomyosin of 0.3.

(b) The stimulation of the actin-activated ATPase activity of gizzard myosin by skeletal muscle F-actin in the absence (Δ , \blacktriangle) and the presence (\square , \blacksquare) of the tropomyosin-kinase fraction. The tropomyosin-kinase fraction was added to give a fixed ratio of tropomyosin to actin of 0.2.

Figure 5(a) shows the effect of the tropomyosin-kinase fraction on actomyosin reconstituted from gizzard myosin and gizzard F-actin at two ratios of actin to myosin. In both cases there is an approximately threefold activation above that observed with no added tropomyosin-kinase fraction.

The effect of the tropomyosin-kinase fraction was also tested on actomyosin reconstituted from myosin and actin from skeletal muscle. In this case a more than twofold activation of the actin-activated ATPase activity took place (Fig. 5(b)). However, in marked contrast to actomyosins containing gizzard myosin this activation was Ca-insensitive. As seen in Figure 5(b) maximal activation was obtained in the range of 150 µg tropomyosin per mg actin, a value corresponding to a molar ratio of tropomyosin to actin of about 1:10. Since no incorporation of radioactive phosphorus into the skeletal muscle myosin light chains could be detected in parallel phosphorylation assays, the noted activation could be attributed solely to the presence of gizzard tropomyosin (see also Discussion).

(c) *Dependence of activation on MgATP concentration*

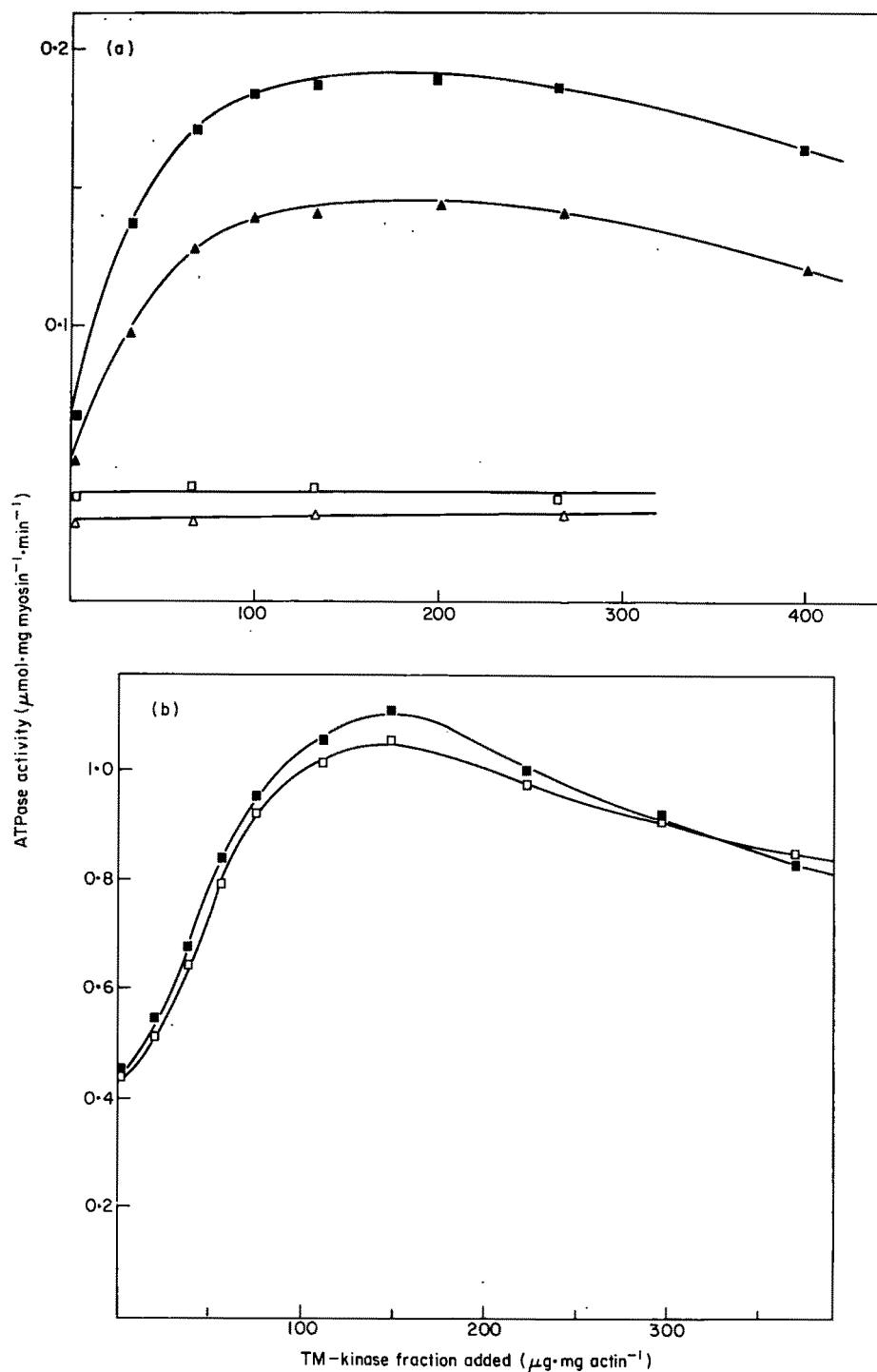
In the case of striated muscle a Ca-insensitive activation of ATPase activity occurs at low MgATP concentrations and this has been attributed to the "switching on" of the thin filaments by rigor link formation (see review by Weber & Murray, 1973). The same effect is not observed in vertebrate smooth muscle. Figure 6 shows the actin-activated ATPase activity for MgAM 25 plus an optimal amount of the tropomyosin-kinase fraction (solid circles) and for MgATP concentrations from 1 µM to 1000 µM. In the absence of Ca no activation is observed and in its presence there is a steady increase in ATPase activity with increasing ATP concentration. For natural actomyosin the same result was obtained (Fig. 6, triangles).

(d) *Binding of tropomyosin to actomyosin*

The sedimentation of tropomyosin-free actomyosin or reconstituted actomyosin in the presence of different amounts of the tropomyosin-kinase fraction followed by subsequent washing of the pellets at low ionic strength showed that the tropomyosin in this fraction was readily bound to the actomyosin complex under the conditions used for the ATPase assays. This is illustrated in Figure 1(h) by polyacrylamide gels of typical actomyosin pellets, in this case corresponding to reconstituted skeletal muscle actomyosin. The specificity of this binding to the actin component, as would be expected, was shown by corresponding experiments in which F-actin was mixed with the tropomyosin-kinase fraction and then collected by high-speed sedimentation (Fig. 1(i)).

(e) *Myosin light-chain kinase preparations*

As indicated in Materials and Methods, preparations were obtained from chicken gizzard that were rich in the myosin light-chain kinase but contained very small amounts of tropomyosin. These preparations corresponded to the supernatant obtained after precipitation of actomyosin with Ca or Mg (Fig. 2(e)) and to the direct extract of myofibrils obtained in the absence of ATP at pH 7.6 and in the presence of 25 mM-MgCl₂ (Fig. 2(h)). Table 1 shows the myosin light-chain kinase activities of these preparations as well as for the various preparations of contractile proteins expressed in relation to the light-chain kinase activity of purified myosin. The highest



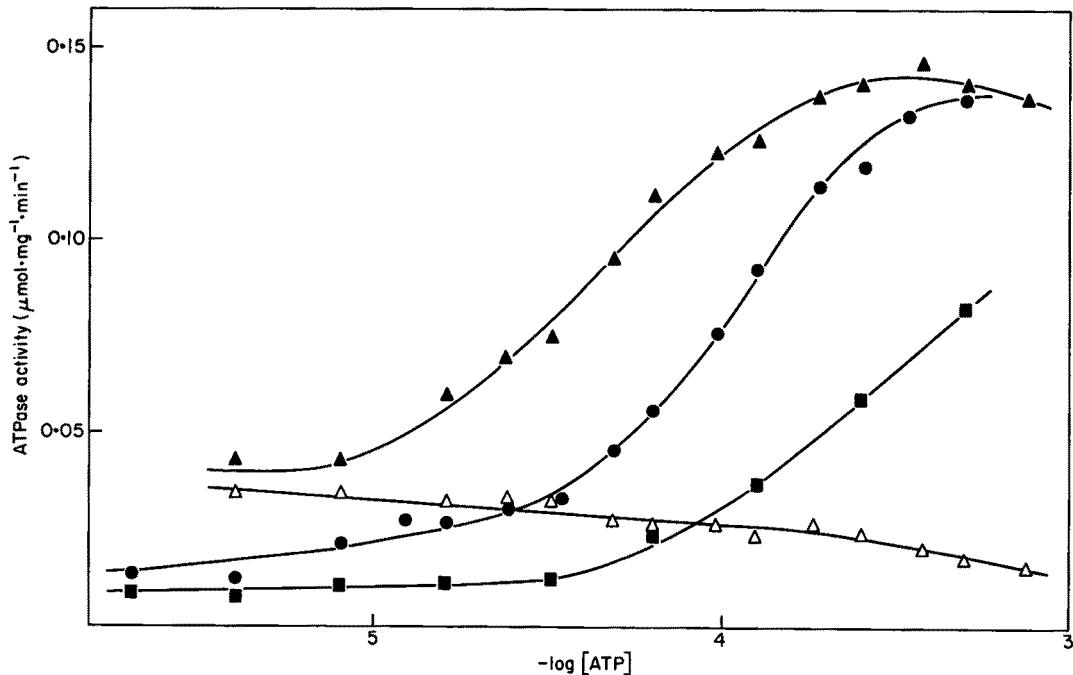


FIG. 6. Dependence of actin-activated ATPase activity on MgATP concentration. (Δ , \blacktriangle) Natural actomyosin. The remaining curves correspond to tropomyosin-free actomyosin to which different amounts of the tropomyosin-kinase fraction were added. For optimal amounts of the tropomyosin-kinase fraction (\bullet) the activities were the same as for normal actomyosin. By reducing the added amount of the tropomyosin-kinase fraction to 1/3 the activity dropped by about 50% (\blacksquare). Note that there is no activation at low MgATP concentrations as observed in skeletal muscle.

activity, at least 200 times that of myosin, was shown by the myofibril extract while the activity of the actomyosin supernatant (Mg or Ca) was about 10 to 15 times less and of the order of that exhibited by the tropomyosin-kinase fraction. For twice-precipitated tropomyosin-free actomyosin (CaAM 25 II) and also for F-actin very little kinase activity could be detected, while thin filaments exhibited about half of the activity shown by myosin.

Partial purification of the light-chain kinase was achieved by gel filtration of the myofibril extract on Sepharose 6B (Fig. 7) or Sephadex G150. Under the conditions employed (60 mM-KCl, 1 mM-MgCl₂, 1 mM-cysteine, 40 mM-imidazole, pH 7.4) the kinase suffered no further loss in activity than could be accounted for by the dilution resulting from the fractionation.

FIG. 5. (a) The stimulation by the tropomyosin-kinase (TM-kinase) fraction of the actin-activated ATPase activity of actomyosin reconstituted from gizzard myosin and F-actin. The data show the activation produced by increasing amounts of the tropomyosin-kinase fraction for 2 different ratios of actin to myosin. The actin to myosin weight ratios were, 1:2 (Δ , \blacktriangle) and 1:1 (\square , \blacksquare).

(b) The stimulation of actin-activated ATPase activity by the tropomyosin-kinase fraction on reconstituted skeletal muscle actomyosin for a weight ratio of actin to myosin of 7:10. In contrast to the effect observed with gizzard myosin this activation is Ca-insensitive.

TABLE 1
Myosin light-chain kinase activity of gizzard preparations

Preparation	Relative kinase activity	Total protein concn range (mg/ml)
Myosin	1	4-6
Mg supernatant of CMF	0.1	3-4
F-actin	0.1	4-6
Thin filaments	0.5	6-8
Last wash of myofibrils	0.1	0.5-1
Ca or Mg actomyosin	6.5	3-5
Ca or Mg supernatant of CAM	15	2-3
Ca or Mg actomyosin 25	0.1	4-6
Tropomyosin-kinase fraction	10	5-7
Kinase extract of myofibrils	200	2-3
Column-purified kinase	100	0-0.2

CMF, crude myosin fraction; CAM, crude actomyosin.

(f) *The separate effects of the myosin light-chain kinase and tropomyosin*

Using the preparations described above that contained very little or no tropomyosin but were rich in the light-chain kinase, together with gizzard tropomyosin prepared by the normal Bailey (1948) procedure (Fig. 2(d)), it was possible to establish the separate effects of these two components on the activation of the actin-myosin interaction.

In the first instance it was found that tropomyosin alone had no activating effect at all on tropomyosin-free actomyosin, MgAM 25 or CaAM 25 (Figs 3 and 8). In contrast, the fractions rich in the myosin light-chain kinase produced a considerable Ca-sensitive activation of the actin-activated ATPase activity (Fig. 8, lower curve). When tropomyosin was added either to the kinase-rich extract or, in a fixed amount, to the tropomyosin-free actomyosin, increased activation was observed (Fig. 8, solid squares and crossed circles, respectively). In these experiments the stimulation produced by tropomyosin was found to be of the order of 40 to 50% above that produced by the crude light-chain kinase fraction alone. But as we have already noted, these fractions contained small amounts of tropomyosin (see gels, Fig. 2(e) and (h)).

As indicated in the previous section, the light-chain kinase could be partially purified by gel filtration without a significant loss in activity and, it was also free of tropomyosin. The separate effects of this partially purified kinase and tropomyosin are shown in Figure 9(a) and (b). These Figures correspond, respectively, to experiments with tropomyosin-free actomyosin (CaAM 25) and actomyosin reconstituted from purified gizzard myosin and F-actin. The lower curve in each case shows the activation produced by increasing amounts of the column-purified kinase in the absence of tropomyosin. In both cases there is a saturation of the actin-activated ATPase activity with increasing amounts of the light-chain kinase.

The upper curves in Figure 9(a) and (b) show the activating effect of increasing amounts of tropomyosin in the presence of a fixed, optimal amount of the column-purified myosin light-chain kinase, as determined from the kinase-activating curves. In the case of both tropomyosin-free actomyosin and reconstituted actomyosin,

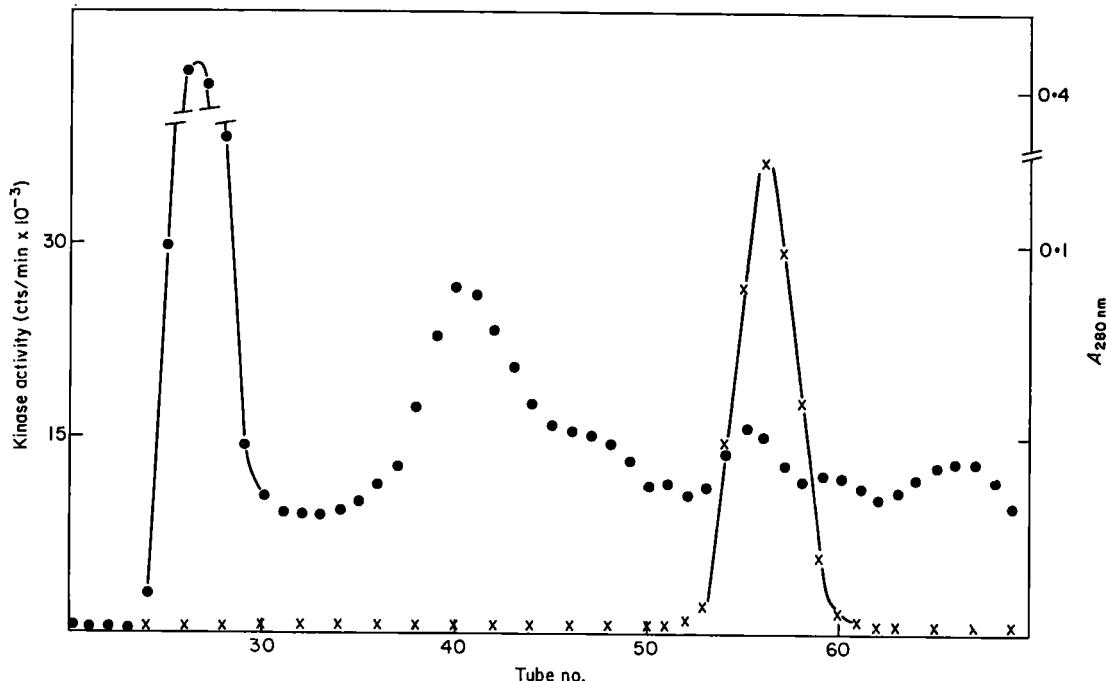


FIG. 7. Gel filtration of the light-chain kinase extract of myofibrils on Sepharose 6B. A 15-ml sample was applied to a 3.4 cm × 95 cm column equilibrated and eluted at 15 ml/h with 0.06 M-KCl, 1 mM-MgCl₂, 1 mM-cysteine, 40 mM-imidazole (pH 7.4) at 4°C. Fractions (120 drops) were assayed for absorption (●) and kinase activity (×).

tropomyosin caused a pronounced two to threefold stimulation of the actin-activated ATPase activity above that produced by optimal amounts of the myosin light-chain kinase alone.

4. Discussion

The results obtained with tropomyosin-free actomyosin show that the stimulation of actin-activated ATPase activity in vertebrate smooth muscle is absolutely dependent on the presence of an endogenous myosin light-chain kinase. Thus, only when myosin is phosphorylated via the light-chain kinase can interaction between myosin and actin take place. These results are fully consistent with the finding that the phosphorylation process is very rapid, reaching saturation under experimental conditions within about 10 seconds (Sobieszek, 1977b) and as such precedes the rise in actin-activated ATPase activity. The conclusion from these findings is that while the control via Ca of the actin-myosin interaction in smooth muscle centres around the myosin molecule (Bremel, 1974; Sobieszek & Small, 1976), this control is further dependent on a specific myosin light-chain kinase. (The fact that this kinase is co-purified with myosin prepared under conditions of low ionic strength (Sobieszek, 1977b) explains the Ca-sensitive responses noted earlier (Sobieszek & Small, 1976) for actomyosins reconstituted from gizzard myosin and F-actin.)

The site of action of Ca²⁺ has yet to be clarified. As we showed previously (Sobieszek & Small, 1976), smooth muscle myosin binds significant amounts of

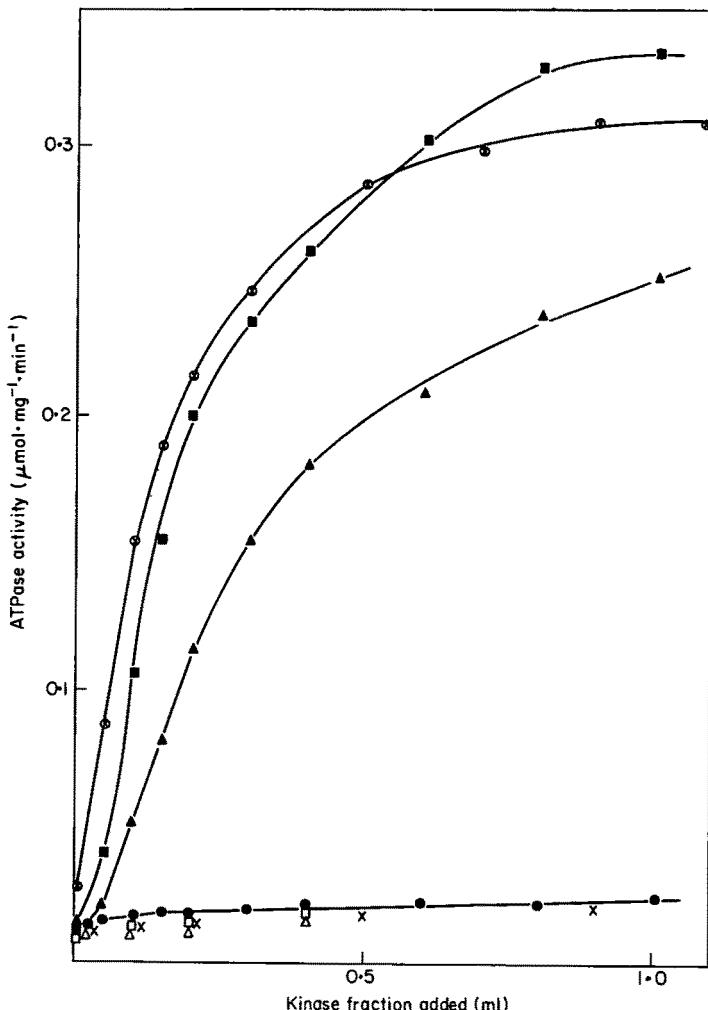


FIG. 8. Activating effect of the crude myosin light-chain kinase preparations (see text) and tropomyosin on the activation of tropomyosin-free actomyosin (CaAM 25).

(△, ▲) The addition of the kinase-rich supernatant obtained after actomyosin precipitation. This supernatant contained a small amount of tropomyosin. (○, ■) The increase in activation produced by dissolving tropomyosin (5 mg/ml) directly in the kinase-rich supernatant prior to addition to the assay tubes. Tropomyosin alone had no activating effect (●).

The effect of the kinase extract of myofibrils on CaAM 25 in the presence of a fixed and optimal amount of tropomyosin is shown by (⊗) for Ca and (×) for EGTA.

Ca^{2+} which may not be accounted for by the relatively low amounts of the light-chain kinase present in purified myosin. In contrast, the isolated light chain, while showing a Ca-dependent phosphorylation does not bind Ca^{2+} . Thus, while the latter result would suggest that Ca^{2+} acts only at the level of the kinase we cannot exclude the possibility that in the intact system Ca acts both on myosin and on the light-chain kinase.

The presence of a myosin light-chain kinase has previously been demonstrated in skeletal and cardiac muscle (Perrie *et al.*, 1973; Frearson & Perry, 1975), and more recently also in vertebrate smooth muscle (Frearson *et al.*, 1976), but a direct link

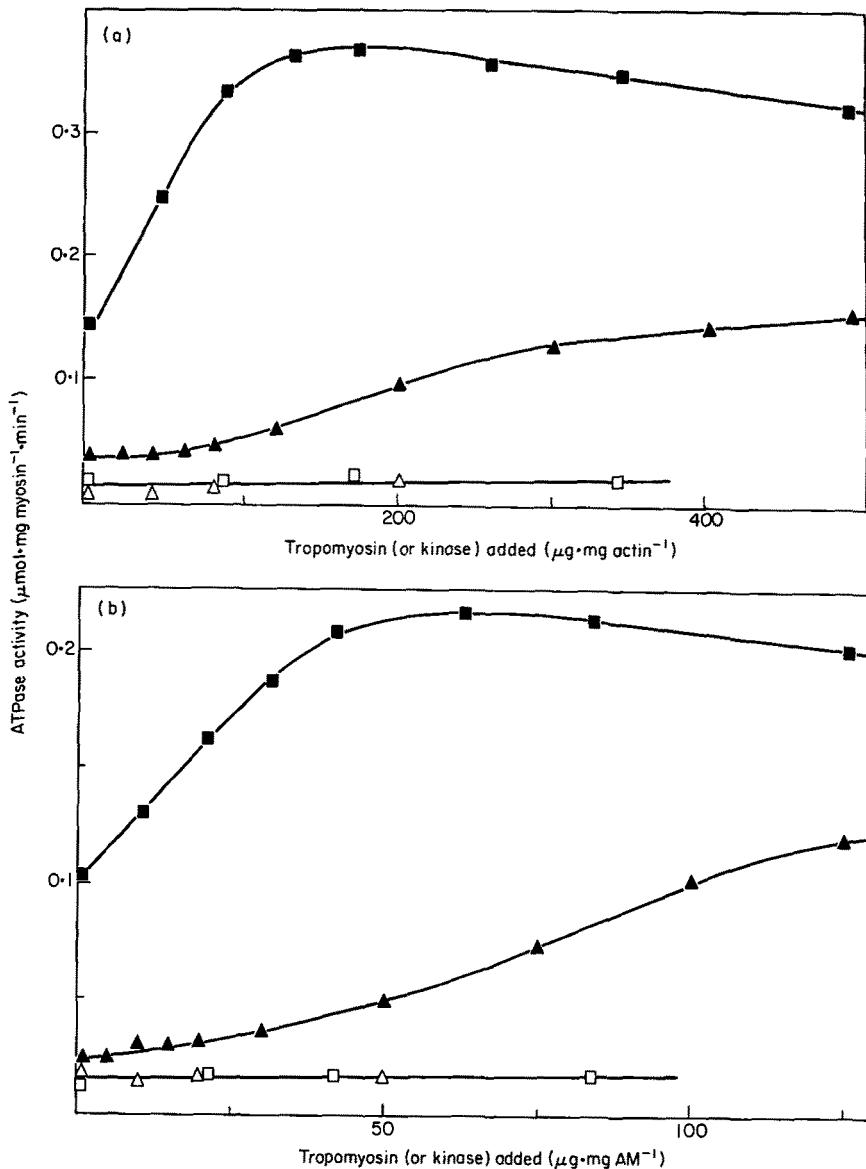


FIG. 9. The separate effects of the light-chain kinase and tropomyosin on reconstituted gizzard actomyosin (a) and tropomyosin-free actomyosin (b). The weight ratio of actin to myosin in (a) was 1:1.

(Δ, ▲) The addition of increasing amounts of the column-purified light-chain kinase (arbitrary units). (□, ■) The activating effect for the same preparation of increasing amounts of tropomyosin in the presence of an optimal and fixed amount of the myosin light-chain kinase.

between this phosphorylation and actin-activated ATPase activity was not found. Following reports by us (Sobieszek, 1977a; Bremel *et al.*, 1977) Aksoy *et al.* (1976) carried out parallel investigations on the phosphorylation of smooth muscle actomyosin. The ATPase activities of the gizzard actomyosin preparations used were, however, of the order of tenfold lower than those reported here and in the range of that obtained for our purified myosin; interpretation of their results is therefore not

as straightforward as ours. A Ca-dependent phosphorylation of actomyosin was recognised and in accord with the present studies these authors found that the kinase co-purified with tropomyosin on ammonium sulphate precipitation and activated the actomyosin ATPase. We should emphasize here that the kinase activities discussed in the present study were considerably higher than those reported elsewhere. In earlier reports it has only been possible to measure light-chain kinase activity with assay times ranging from several minutes to several hours. In contrast, we have found that saturation of phosphate incorporation with crude or column-purified kinase, using 0.5 mg of the L₂₀ light chain/ml as substrate, occurs within one to three seconds. On consideration of the time-course of the rise in actin-activated ATPase activity (Sobieszek, 1977b) such light-chain kinase activities correlate well with a role associated with the activation of the actomyosin system.

A myosin phosphorylation, specifically of the L₂₀ light chain of myosin, was noted some time ago in blood platelets (Adelstein *et al.*, 1973) and in this system it was shown, in recombination experiments with skeletal muscle F-actin, to be associated with a higher actin-activated ATPase activity (Adelstein & Conti, 1975). The platelet kinase responsible for this phosphorylation was further shown to be capable of phosphorylating smooth muscle myosin as well as certain non-muscle myosins (Adelstein & Conti, 1976), but in contrast to the smooth muscle enzyme and those from striated muscle (Perrie *et al.*, 1973) its activity was apparently Ca-independent. In consequence definitive evidence for the involvement of the light-chain phosphorylation in the Ca-regulation process was not obtained.

The complete dependence of the activation of vertebrate smooth muscle actomyosin on the myosin light-chain kinase contrasts markedly with the situation in striated muscle. In recombined striated muscle myosin and F-actin, as well as in myofibrils, normal activation took place in the absence of any detectable light-chain phosphorylation. We may also add that like Frearson *et al.* (1976) we found the smooth muscle light-chain kinase to be incapable of phosphorylating the skeletal muscle 19,000 molecular weight) light chain. The significance of the light-chain phosphorylation in striated muscle where Ca-regulation is associated with the thin filaments is as yet unclear. However, it does not seem unlikely that the skeletal muscle phosphorylatable light chain may serve some kind of modulatory function which has hitherto been overlooked (Perrie *et al.*, 1973; Kendrick-Jones, 1974).

How the mechanism of activation in vertebrate smooth muscle is related to that obtaining in other contractile systems where Ca-regulation is myosin-linked has yet to be established. Present evidence would suggest that a similar phosphorylation of a myosin light chain is not a feature of Ca-activation in invertebrate muscles regulated *via* myosin (Kendrick-Jones, personal communication; Frearson *et al.*, 1976). We observed, consistent with these findings, that tropomyosin removal from scallop actomyosin (under conditions in which both the kinase and tropomyosin were removed from vertebrate smooth muscle) resulted in a tropomyosin-free actomyosin of which the Ca-sensitivity was essentially unchanged, as compared to normal scallop actomyosin. (Sobieszek & Small, unpublished data).

Mrwa & Rüegg (1975) have reported that the ATPase activity of actomyosin reconstituted from smooth muscle myosin S-1 and striated muscle F-actin exhibits a Ca-sensitivity of the order of 50%. In our own experiments we observed that this system was, in contrast, Ca-insensitive (Sobieszek & Small, 1976) and we showed further that the L₂₀ light chain of myosin was degraded under the conditions used to

produce smooth muscle myosin S-1. We also observed that a proteolytic degradation product of the heavy chains of myosin, present in S-1, migrated on polyacrylamide gels in a position very close to that characteristic for the L₂₀ light chain, giving the impression that the L₂₀ light chain was preserved in this subfragment. While further experiments will be required to clarify the discrepancy in the results obtained we note that these authors have not reported that the data has been corrected for the activity of S-1 alone in the presence and absence of Ca.

The requirement for myosin phosphorylation in the process of the activation of the ATPase activity of smooth muscle actomyosin suggests that there is a converse process, namely dephosphorylation, which effects inactivation, or "relaxation". Since neither an inactivation nor a dephosphorylation is produced by the removal of Ca ions from active, phosphorylated gizzard actomyosin (Sobieszek, 1977a), we conclude that this is effected by a phosphatase acting on the light chain. Such an enzyme, specific for the 20,000 molecular weight light chain of myosin has recently been isolated from striated muscle (Morgan *et al.*, 1976) and has been shown to be active in the absence of Ca ions. Our more recent experiments have shown a corresponding enzyme to be present in the "kinase extract" of gizzard myofibrils, but not in purified actomyosin, and have demonstrated that this enzyme does effect an inactivation of phosphorylated actomyosin in the absence of Ca. These results will be presented in detail elsewhere (Small & Sobieszek, unpublished data).

As well as demonstrating the dependence of the actin-myosin interaction in vertebrate smooth muscle on the myosin light-chain kinase we have also shown that tropomyosin plays a significant role in the activation process. Only in the presence of this protein is full activation of the actin-activated ATPase activity achieved, the activity in the presence of tropomyosin being two to threefold greater than in its absence. A similar activation by tropomyosin was noted by Lehman & Szent-Györgyi (1972) in *Limulus* where Ca-regulation operates *via* the thin filaments, and was there shown to be associated with a higher affinity of the tropomyosin-containing thin filaments for *Limulus* myosin. Apart from an early report by Katz (1964) the same effect has not been generally observed in vertebrate skeletal muscle but an activation by tropomyosin has been demonstrated to occur in this system at low concentrations of MgATP (Bremel & Weber, 1972; Bremel *et al.*, 1973; Shigekawa & Tonomura, 1972, 1973). In the present study smooth muscle tropomyosin caused an approximately 2.5-fold activation of the ATPase activity of reconstituted actin and myosin from skeletal muscle even at "high" MgATP concentrations (1 mM), suggesting a different effect of this tropomyosin as compared to its skeletal muscle counterpart. But, in addition, we have also observed (unpublished observations) that the activation by smooth muscle tropomyosin is further increased by about 50%, at lower MgATP concentrations (between 60 and 120 µM) and thus mimics, in this region, the activation noted with skeletal muscle tropomyosin (Bremel *et al.*, 1973). We thus conclude that in smooth muscle, tropomyosin may act as in skeletal muscle (see review by Weber & Murray, 1973) to effect a co-operative change in the actin filament, on activation, which facilitates actin-myosin interaction and causes a potentiation of actin-activated ATPase activity.

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