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Non-correlation of phosphorylation of the P-light chain and the actin activation of the ATPase of chicken gizzard myosin

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1. The enzymic properties of myosin isolated from chicken gizzard by three different methods have been compared. 2. Although the specific Ca^{2+}-stimulated ATPases of all preparations were similar and high, there were significant differences in the specific activities of the Mg^{2+}-stimulated actomyosin ATPases. 3. There was no direct correlation between the Mg^{2+}-stimulated actomyosin ATPase activity and the extent of P-light-chain phosphorylation in any of the three myosin preparations. 4. A fraction that activates the Mg^{2+}-stimulated actomyosin ATPase of gizzard muscle has been isolated from a gizzard muscle filament preparation. 5. The activator was specific for the Mg^{2+}-activated actomyosin ATPase of smooth muscle. 6. The activator required the addition of calmodulin for full effect.

It is a widely held view that, in resting vertebrate smooth muscle, the P-light chain of myosin is mainly, if not entirely, dephosphorylated (Somlyo *et al.*, 1981), in which state the Mg^{2+}-stimulated myosin ATPase cannot be activated by actin. On stimulation of smooth muscle, the ensuing rise in intracellular Ca^{2+} concentration activates the myosin light-chain kinase, the P-light chain is phosphorylated, which in turn activates the actomyosin ATPase, resulting in cross-bridge cycling and contraction. According to this mechanism, relaxation occurs when the light-chain kinase is inactivated by the fall in Ca^{2+} concentration to the resting level and the P-light chain is dephosphorylated by myosin light-chain phosphatase.

This hypothesis is based on investigations from several laboratories indicating that the Mg^{2+}-stimulated ATPase of preparations of smooth-muscle actomyosin is relatively inactive when the P-light chain is dephosphorylated, but becomes active when the system is incubated with ATP and endogenous or added myosin-light-chain-kinase preparations (Sobieszek & Small, 1976; Gorecka *et al.*, 1976; Chacko *et al.*, 1977). Despite these findings there are persistent reports in the literature of activation of the Mg^{2+}-stimulated ATPase of smooth-muscle actomyosin that does not correlate with the level of P-light-chain phosphorylation (Ebashi, 1976; Cole *et al.*, 1980). Indeed Ebashi and his collaborators (Mikawa *et al.*, 1977) consider that smooth-muscle actomyosin ATPase is regulated by a system of unique proteins, leiotonin A and C, whereas Marston and his collaborators (Marston *et al.*, 1980; Walters & Marston, 1981) have evidence for a different regulatory system involving phosphorylation of a protein of mol.wt. 21000 associated with the I-filament.

This controversy has not been resolved by studies of the changes in the state of phosphorylation of the P-light chain during contraction of intact smooth-muscle preparations. In general, phosphorylation increases on stimulation (Janis *et al.*, 1981; Somlyo *et al.*, 1981), but in some cases at least the correlation between phosphorylation and contractile activity is not as complete as would be expected from the original hypothesis that postulates a simple phosphorylation–dephosphorylation process activating the cross-bridge cycle (Kamm *et al.*, 1981; Murray & England, 1980).

The persistent inconsistencies in the experimental data suggest that the system regulating the actomyosin ATPase of smooth muscle is more complex than is currently realized. In attempts to explain the apparently contradictory results obtained from a number of laboratories regarding the role of P-light-chain phosphorylation, we have undertaken a systematic study of the enzymic properties of myosin prepared by several different methods. From this investigation we conclude that a protein factor can be isolated from smooth-muscle myosin preparations that strongly activates the actomyosin ATPase in a manner that is not correlated with the degree of phosphorylation of the P-light chain.

Materials and methods

Materials

Chicken gizzards were gifts of the Halal Meat

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Company, Rubery, Birmingham, U.K., and Poultry Packers, Pershore, Worcs., U.K. Acrylogel and acrylamide were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. and [γ-^{32}P]ATP from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of myosin E and myosin AS

Crude actomyosin. All procedures were carried out at 4°C. Fresh chicken gizzards were stripped of fat and the inner, yellow membranous layer, washed, minced, then homogenized twice in 10 vol. of 16.8 mM-Na_2HPO_4/3.2 mM-NaH_2PO_4/1 mM-EDTA/0.1 mM-dithiothreitol/streptomycin (0.1 g/litre)/3 mM-NaN_3/0.5 mM-phenylmethanesulphonyl fluoride adjusted to pH 7.5 at 4°C with 1 M-NaOH. The residue was rehomogenized and stirred gently for 1 h in 3 vol. of 16.8 mM-Na_2HPO_4/3.2 mM-NaH_2PO_4/0.5 M-KCl/2 mM-$MgCl_2$/1 mM-EGTA/1 mM-ATP/1 mM-dithiothreitol/0.5 mM-phenylmethanesulphonyl fluoride/3 mM-NaN_3/streptomycin (0.1 g/litre), pH 7.5, at 4°C, then centrifuged for 30 min at 4000 *g* and the supernatant diluted into 10 vol. of 15 mM-β-mercaptoethanol. After 30 min the precipitate was collected by centrifugation for 10 min at 5000 *g*, redissolved in 0.3 M-NaCl by the addition of 3 M-NaCl and then diluted with 20 vol. of 15 mM-β-mercaptoethanol/1 mM-$NaHCO_3$/2 mM-NaN_3; after 30 min the crude actomyosin was collected by centrifugation for 20 min at 5000 *g*.

Myosin E. Crude actomyosin (10 mg/ml) was suspended in a medium of final concentration 8.4 mM-Na_2HPO_4/1.6 mM-NaH_2PO_4/100 mM-$MgCl_2$/5 mM-NaATP/1 mM-dithiothreitol, pH 7.5, for 20 min and centrifuged for 1 h at 15000 *g*. The preparation was continued as described by Ebashi (1976), with the addition of 1 mM-dithiothreitol throughout (Fig. 1*a*).

Myosin AS. Myosin AS was obtained from the crude actomyosin by a modification of the method of Katoh & Kubo (1977). The first stage of the preparation was the same as for myosin E, apart from the addition of 0.25 M-NaCl to the buffer. The supernatant solution was fractionated with saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.0 with 1 M-NH_3. The precipitate obtained between 20 and 50% saturation was dialysed at least 4 h against 20 vol. of 0.25 M-KCl/1 mM-EDTA/20 mM-Tris/1 mM-EDTA/1 mM-dithiothreitol/3 mM-NaN_3, adjusted to pH 7.5 at 4°C with 1 M-HCl, and centrifuged for 1 h at 15000 *g*. The supernatant, including partially sedimented gel-like material, was mixed with 4 vol. of 1 mM-$NaHCO_3$/1 mM-dithiothreitol/2 mM-NaN_3, and after 30 min the precipitated myosin was collected by centrifugation for 15 min at 25000 *g* (Fig. 1*d*).

Preparation of myosin SL

Myosin SL was prepared by a method similar to that of Sobieszek (1977). Gizzard muscle was dissected from both membranes, minced then washed six times in 5 vol. of 60 mM-KCl/1 mM-$MgCl_2$/15 mM-mercaptoethanol/20 mM-imidazole buffer (adjusted to pH 6.8 with 1 M-HCl)/streptomycin (100 mg/litre)/3 mM-NaN_3/0.5 mM-phenylmethanesulphonyl fluoride with the addition of Triton X-100 (0.5%) in the second and third washes. The first five washes were carried out by homogenizing in a Waring blender for about 20 s, the sixth in a glass Potter-type homogenizer, followed in each case by centrifugation for 15 min at 2000 *g*. The sedimented washed filament preparation was mixed with an equal volume of glycerol and stored at −20°C.

The filament preparation was washed twice again by centrifugation in the original wash buffer immediately before actomyosin was extracted by homogenization of the stored filaments in a Potter-type homogenizer with 5 vol. of a solution containing 10 mM-ATP/2 mM-EGTA/2 mM-EDTA/streptomycin (100 μg/litre)/3 mM-NaN_3, the pH of which was adjusted to 7.2 by addition of 2 M-imidazole. The suspension was centrifuged for 20 min at 75000 *g* and the sediment, the 'initial residue', (Fig. 1*i*) retained for preparation of the activating factor (see the Results section). The pH of the supernatant was re-adjusted to 7.2 with 2 M-imidazole and centrifuged for 4.5 h at 125000 *g* to give the 'second residue' (Fig. 1*j*), which contained some activator, but was usually discarded. The myosin was precipitated from the supernatant by the slow addition of 1 M-$MgCl_2$ to give a final concentration of 35 mM-$MgCl_2$. After being left for a minimum of 30 min it was collected by centrifugation for 30 min at 50000 *g* and dissolved by the addition of 3 M-KCl such as to adjust the concentration to 0.6 M. The myosin was diluted to 0.05 M-KCl, the pH being adjusted to 7.0 with 2 M-imidazole if necessary. Usually it was necesary to add $MgCl_2$ to a final concentration of 10 mM to precipitate the myosin (Fig. 1*c*), which was stored as a concentrated suspension in 10 mM-$MgCl_2$/0.05 M-KCl after centrifugation for 20 min at 2000 *g*.

Preparation of other muscle proteins

Actin was prepared from an acetone-dried fibre of rabbit skeletal muscle and chicken gizzard by the method of Hartshorne *et al.* (1967) or Spudich & Watt (1971). Tropomyosin was prepared from pig stomach or chicken gizzard muscle by the method of Cummins & Perry (1973).

Preparation of myosin light-chain kinase and myosin light-chain phosphatase

Myosin light-chain phosphatase was a partially purified preparation obtained from chicken gizzard muscle by the procedure described by Morgan *et al.* (1976) taken up to the stage of gel filtration. Myosin

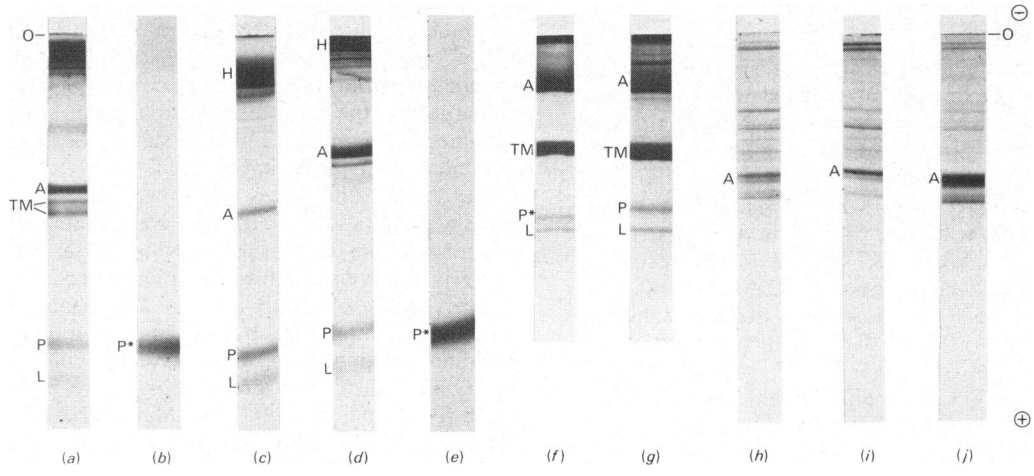

Fig. 1. *Electrophoresis of gizzard myosin and activator preparations*

Electrophoresis was carried out in sodium dodecyl sulphate in Tris/borate, pH 6.0 (*a* and *d*), or Tris/Bicine, pH 8.0 (*b* and *h–j*), or in 8 M-urea, pH 8.6 (*f* and *g*). See the Materials and methods section for details. H, Myosin heavy chain; P, P-light chain; P*, phosphorylated P-light chain; L, 17000-dalton light chain; A, actin; TM, tropomyosin; O, origin. (*a*) Myosin E (100 μg) incubated 5 min under the conditions described in Fig. 2. (*b*) Autoradiograph of gel illustrated in (*a*). (*c*) Myosin SL (100 μg). (*d*) Myosin AS (70 μg) incubated 5 min under conditions as described in Fig. 2. (*e*) Autoradiograph of gel illustrated in (*d*). (*f*) Myosin AS (90 μg) incubated under the conditions described in Fig. 3 for 6 min in absence of added phosphatase. (*g*) Myosin AS (90 μg) treated as for (*e*), but incubated in the presence of gizzard phosphatase (Fig. 3). (*h*) Activator preparation precipitated between 40 and 60% saturation of $(NH_4)_2SO_4$ (about 75 μg). (*i*) 'Initial residue' of myosin SL preparation after centrifugation for 20 min at 75000 *g* (about 75 μg). (*j*) 'Second residue' of myosin SL preparation after centrifugation for 4.5 h at 125000 *g* (about 75 μg).

light-chain kinase was prepared from rabbit skeletal muscle by the method of Pires & Perry (1977) also taken up to the gel-filtration stage, although on some occasions the purified enzyme was used. Calmodulin was prepared from bovine brain either by the method of Watterson *et al.* (1976) or by the organic-solvent method of Grand *et al.* (1979).

ATPase assays

Normally the method used was an adaptation of that of Schaub & Perry (1969). Up to 2 mg of myosin were assayed at 25 or 30°C for intervals of up to 40 min in a total vol. of 1 ml containing 2.5 mM-NaATP (50 mM stock solution adjusted to pH 7.5 with 0.5 M-NaOH)/2.5 mM-$MgCl_2$/2.5 mM-dithiothreitol (or dithioerythritol)/50 mM-Tris/HCl (500 mM stock solution adjusted to pH 7.5 with 1 M-HCl). For the Ca^{2+}-stimulated ATPase the $MgCl_2$ was replaced with 2.5 mM-$CaCl_2$, with and without 0.3 M-NaCl. Rabbit skeletal or chicken gizzard actin (1 mg/ml) and pig stomach or chicken gizzard tropomyosin (75 μg/ml) were added as indicated. Phosphate was determined by the method of Fiske & SubbaRow (1925) in a final volume of 10 ml.

Occasionally ATPase activity was monitored by ^{32}P release from [γ-^{32}P]ATP (0.2–1.0 Ci/mol), other conditions being the same as above. The ^{32}P release was estimated in 200 μl samples, which were added to 1 ml of cold 2.5% acid-washed Norit A in 2%-$HClO_4$/50 mM-NaH_2PO_4, filtered through Whatman no. 1 filter paper and ^{32}P in the filtrate measured by Čerenkov scintillation counting.

Incorporation of ^{32}P into myosin

This was determined in the reaction mixtures used for the assay of ATPase activity by the measurement of ^{32}P liberated from [γ-^{32}P]ATP. Portions (0.75 ml) were pipetted into 2 vol. of bovine serum albumin (1 mg/ml) in 0.6 M-KCl at 0°C and 1 ml of 15% trichloroacetic acid was added immediately. Covalently bound ^{32}P was then determined as described by Perry & Cole (1974).

Myosin light-chain kinase and myosin light-chain phosphatase assays

Kinase assays were carried out at 30°C as described by Nairn & Perry (1979) and phosphatase assays as described by Morgan *et al.* (1976), except that in the latter case the final volume was 0.2 ml. In both assays the concentration of skeletal light chains was maintained at about 100 times that of the myosin preparations being assayed.

Electrophoresis

Polyacrylamide-gel electrophoresis in one dimension was performed in vertical (6 cm × 20 cm × 0.3 cm) slab assemblies as described by Perrie *et al.* (1973). The procedures used were either those described by Schaub & Perry (1969), who used 12.5%-Acrylogel gels with 8 M-urea/20 mM-Tris/125 mM-glycine buffer, pH 8.6, in the gels, or as described by Weber & Osborn (1969), on 10% (w/v)-polyacrylamide gels in 83 mM-Tris/400 mM-boric acid/0.5% (w/v) sodium dodecyl sulphate, pH 6.0, or in 0.1 M-Tris/0.1 M-Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/1% (w/v) sodium dodecyl sulphate, pH 8.3. Two-dimensional electrophoresis was carried out by the method of Anderson & Anderson (1978*a*,*b*).

The relative amounts of bands and spots on the gels was determined with a Zeineh soft-laser scanning densitometer with integrator (Helena Laboratories). Radioactive proteins were located on stained polyacrylamide gels either by autoradiography (Moir *et al.*, 1977) or by scintillation counting of gel slices (Perry & Cole, 1974).

Results

Differences in myosin preparations isolated from crude chicken gizzard actomyosin

Although myosin E and myosin AS (see the Materials and methods section) contained some actin, their Mg^{2+}-stimulated ATPase specific activities were increased 100 and 40% respectively by added skeletal actin. When assayed in the presence of excess actin, myosin AS had a higher specific activity than myosin E isolated from the same crude actomyosin preparation (Table 1).

As both preparations of myosin had similar low Mg^{2+}-stimulated actomyosin ATPase activities in the presence of EGTA, the Ca^{2+}-sensitivity of the ATPase of actomyosin AS, calculated as follows:

$$\text{Calcium sensitivity} = \frac{Mg^{2+}\ \text{ATPase} + \text{EGTA}}{Mg^{2+}\ \text{ATPase} + CaCl_2} \times 100$$

was much greater than that of actomyosin E (Table 1). In contrast with the differences in the Mg^{2+}-stimulated actomyosin ATPase activities of the two preparations, the Ca^{2+}-stimulated myosin ATPase activities in the presence of high salt concentrations were much more comparable (Table 1).

No obvious differences were observed in the two myosin preparations when they were examined by one- or two-dimensional polyacrylamide-gel electrophoresis under dissociating conditions (Figs. 1*a* and 1*d*). Myosin heavy chain and two light chains were the major components, but significant amounts of actin and tropomyosin were present in both preparations. When both preparations were preincubated for 20 min at 25°C with Mg[γ-^{32}P]ATP and light-chain kinase before polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, most of the ^{32}P was located in a band of M_r 20000 (Figs. 1*b* and 1*e*). The identification of this band as the phosphorylated form of the P-light chain was confirmed by two-dimensional electrophoresis, in that ^{32}P was mainly incorporated into a new spot more negatively charged than, but of M_r similar to that of, the spot corresponding to the unphosphorylated P-light chain, which had almost disappeared after incubation with the enzyme. There was no evidence that the light chains were degraded in fresh preparations of myosin E or myosin AS. The dephosphorylated P-light-chain band reappeared on prolonged incubation of phosphorylated myosins (at 25°C) or on storage in the cold, indicating the presence of endogenous myosin light-chain phosphatase in both preparations. Kinase activity was usually higher, and phosphatase activity was usually lower, in myosin AS than in myosin E when measured by the incorporation or loss of ^{32}P from excess added skeletal myosin light chains (Table 2).

Effect of phosphorylation on the actin-activated myosin ATPase

The P-light chains of freshly prepared myosin E and myosin AS were dephosphorylated due to endogenous light-chain phosphatase activity (Table

Table 1. *ATPase activities of chicken gizzard myosin prepared by different methods*

Assays were carried out at 30°C as described in the Materials and methods section. Myosin ATPase assays were carried out on the protein as prepared. For actomyosin ATPase assays, 1 mg of skeletal actin was added per mg of myosin. Results are means ± S.E.M. (*n*).

	Specific activity (nmol/min per mg of myosin)			
	Myosin (2.5 mM-$CaCl_2$)		Actomyosin (2.5 mM-$MgCl_2$)	
Type of preparation	No addition	NaCl (0.3 M)	$CaCl_2$ (0.25 mM)	EGTA (2 mM)
Modified Ebashi (1976) (myosin E)	24 ± 3 (18)	114 ± 15 (20)	22 ± 2 (22)	12 ± 2 (18)
Modified Kato & Kuboh (1977) (myosin AS)	16 ± 12 (12)	148 ± 15 (16)	62 ± 8 (17)	11 ± 1 (17)
Modified Sobieszek (1977) (myosin SL)		179 ± 25 (7)	19 ± 3 (17)	4 ± 1 (17)

Table 2. *Myosin light-chain kinase and phosphatase activities of myosin preparations*

Both enzymes were assayed at 30°C in 50mM-Tris/HCl (pH 7.5)/2.5mM-dithiothreitol/12.5mM-$MgCl_2$, to which was added fast-skeletal-muscle myosin light chains (3mg/ml), prephosphorylated with [γ-^{32}P]-ATP (2Ci/mol) and skeletal-muscle light-chain kinase for the phosphatase assay, and 2mM-[γ-^{32}P]-ATP (1Ci/mol) and skeletal-muscle myosin light chains (10mg/ml) for the kinase assay. Except for the single determination (*), results are means ± S.E.M. (*n*).

	Enzymic activity (nmol/min per mg of myosin)	
Myosin	Myosin light-chain kinase	Myosin light-chain phosphatase
E	10 ± 3 (8)	9 ± 4 (8)
AS	17 ± 4 (10)	4 ± 2 (9)
SL	5*	Trace activity

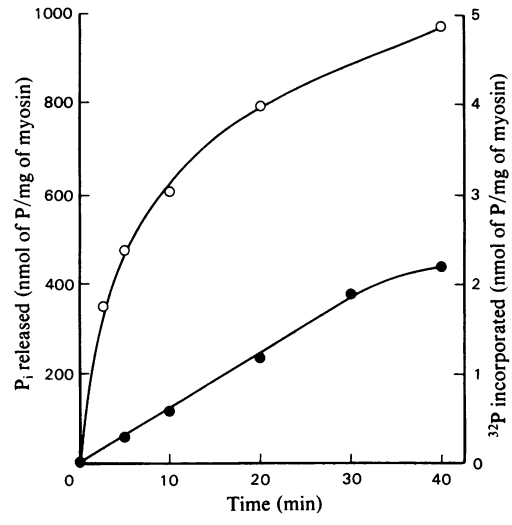

Fig. 2. *Effect of phosphorylation of chicken gizzard myosin on the Mg^{2+}-stimulated ATPase of actomyosin E*

Chicken gizzard myosin E (0.77mg/ml) was incubated at 25°C in 50mM-Tris/HCl (adjusted to pH 7.5 with 1M-HCl)/2.5mM-$MgCl_2$/0.125mM-$CaCl_2$/2.5mM-dithiothreitol/50mM-KCl/2.5mM-[γ-^{32}P]NaATP (0.6Ci/mol) with smooth-muscle tropomyosin (0.1mg/ml), skeletal actin (0.4mg/ml), calmodulin (0.01mg/ml) and light-chain kinase (0.03mg/ml). ●, Mg^{2+}-ATPase activity; ○, myosin phosphorylation.

2). During the course of assaying the Mg^{2+}-activated ATPase, the P-light chain became phosphorylated by the action of endogenous myosin light-chain kinase. The rate of the phosphorylation of myosin E by endogenous enzyme was slow enough to enable a comparison to be made between the extent of P-light-chain phosphorylation and the activity of the actin-activated Mg^{2+}-stimulated ATPase. Phosphorylation of the P-light chain of myosin E, as determined by the amount of ^{32}P incorporated into the myosin, approached completion towards the end of a 40min incubation with [γ-^{32}P]ATP. The activity of Mg^{2+}-stimulated ATPase, determined in the incubation mixture used to monitor the extent of phosphorylation, was unchanged over this period (Fig. 2).

Owing to the relatively high endogenous myosin light-chain kinase activity (Table 2), the P-light chain of myosin AS was usually fully phosphorylated within 1min of the addition of ATP, making it difficult to determine whether a rapid activation of the Mg^{2+}-stimulated ATPase associated with this phosphorylation had occurred, thus accounting for the apparent high steady rate of ATPase. Nevertheless, dephosphorylation of the P-light chain by the addition to the incubation mixture of a preparation of a partially purified myosin light-chain phosphatase from chicken gizzard did not significantly change the rate of the Mg^{2+}-stimulated ATPase of myosin AS (Fig. 3). Even when the phosphatase was added in excess, so that the P-light chain was maintained in the dephosphorylated state, that activity of the ATPase was very similar to that of the control myosin in which the P-light chain was fully phosphorylated. In this experiment the state of P-light-chain phosphorylation throughout the incubation was monitored by polyacrylamide-gel electrophoresis in Tris/glycine/urea buffer (Figs. 1*f* and 1*g*) (see the Materials and methods section).

Effect of centrifugation on the actin-activated ATPase of gizzard myosin

The studies described above suggested that preparations of smooth myosin obtained by different procedures contained other components that might be responsible for the differences in Ca^{2+}-dependent actomyosin Mg^{2+}-stimulated ATPase activities, the action of which did not depend on the extent of phosphorylation of the P-light chain. During evaluation of the procedure described by Sobieszek (1977) for the preparation of myosin, it was noted that after short periods of centrifugation of the final extract, preparations of myosin with relatively high actin-activated ATPase (100–200nmol of P liberated/min per mg of protein) were obtained. These preparations, like myosin E and myosin AS, were not completely free of actin and tropomyosin as judged by electrophoresis. The Mg^{2+}-stimulated ATPase activity measured in the presence of excess actin was calcium-sensitive. After centrifugation at 120000*g* for 4h, preparations of

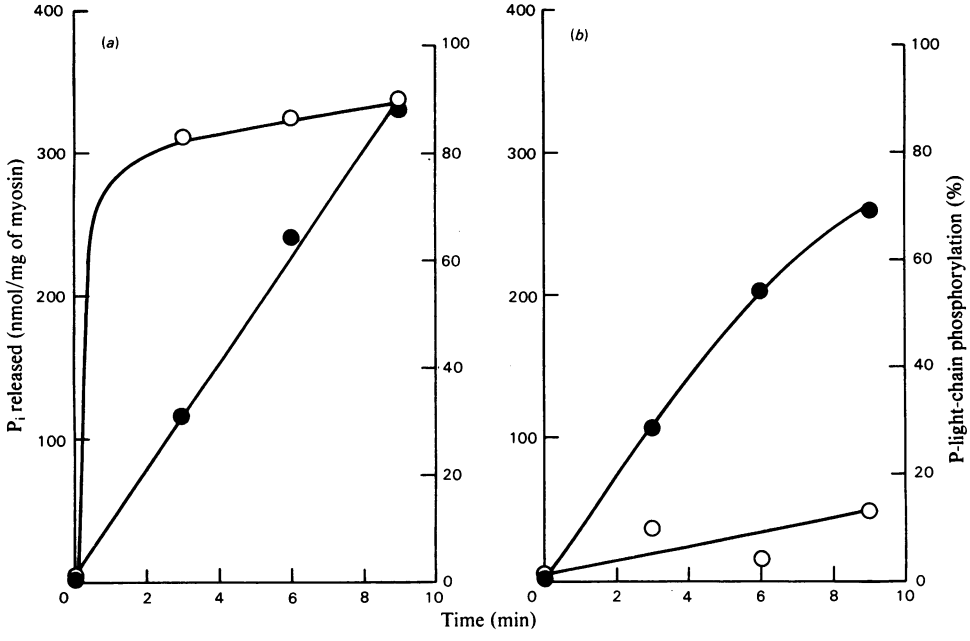

Fig. 3. *Effect of dephosphorylation of the P-light chain on the Mg^{2+}-stimulated ATPase of actomyosin AS*
Chicken gizzard myosin AS (1.8 mg/ml) was incubated at 30°C in 50 mM-Tris/HCl (pH 7.5)/2.5 mM-Mg^{2+}/0.1 mM-Ca^{2+}/5 mM-dithiothreitol/2.5 mM-NaATP (pH 7.5) with calmodulin (5 μg/ml), smooth-muscle tropomyosin (50 μg/ml) and skeletal actin (1 mg/ml). At the times indicated, samples were taken for the determination of ATPase activity by P_i release and the extent of P-light-chain phosphorylation by densitometric scanning of stained polyacrylamide gels obtained by electrophoresis in a Tris/glycine/urea system. (*a*) No further additions; (*b*) partially purified chicken gizzard light-chain phosphatase (0.4 mg/ml) added. ○, P-light-chain phosphorylation. ●, Mg^{2+}-ATPase activity.

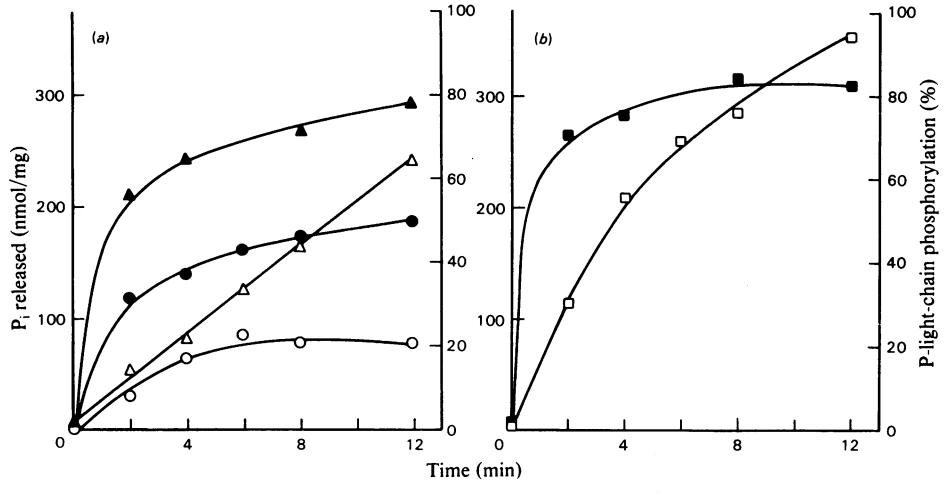

Fig. 4. *Effect of phosphorylation of the P-light chain on the Mg^{2+}-stimulated ATPase of actomyosin S*
Myosin S (1.3 mg/ml) was incubated at 30°C in 50 mM-Tris/HCl (pH 7.5)/5 mM-$MgCl_2$/0.125 mM-$CaCl_2$/5 mM-NaATP/5 mM-dithiothreitol with calmodulin (10 μg/ml), chicken gizzard tropomyosin (0.1 mg/ml) and skeletal actin (1.0 mg/ml). Samples were taken at the time indicated, either into 2 vol. of cold 10 M-urea or into 0.5 vol. of cold 15% (w/v) trichloroacetic acid for determination of light-chain phosphorylation by polyacrylamide-gel electrophoresis and phosphate release respectively. Open symbols, Mg^{2+}-ATPase; closed symbols, P-light-chain phosphorylation. (*a*) ○ and ●, no addition. △ and ▲, activator (60 μg/ml); (*b*) □ and ■, activator (0.17 mg/ml).

myosin were obtained with very much lower actin-activated ATPase activity. These preparations contained very little actin and tropomyosin as judged by electrophoresis (Fig. 1*c*). By taking advantage of this observation a procedure was developed that consistently gave a preparation of myosin that had low Mg^{2+}-stimulated ATPase activity (Table 1) in the presence of either skeletal- or smooth-muscle actin; this we refer to as 'myosin SL' (see the Materials and methods section). The Mg^{2+}-stimulated ATPase of actomyosin preparations made from myosin SL was Ca^{2+}-sensitive. The P-light chains of freshly prepared myosin SL were usually 20–40% phosphorylated. During ATPase assays in the presence of excess actin, the phosphorylation steadily increased to about 50%, but the ATPase activity did not increase (Fig. 4).

Preparation of activating factor

The presence of an activator in myosin preparations of high endogenous actomyosin ATPase activity (50–200nmol of P/min per mg) that was implied by the centrifugation studies was confirmed by the extraction of such a fraction from these preparations. Addition of low-ionic-strength [50mM-Tris/HCl (pH 7.5)/1mM-$MgCl_2$] extracts of freeze-dried myosin preparations with high actomyosin ATPase activity increased the actin-activated ATPase of the myosin SL up to three times.

Examination of various fractions obtained during the preparation of myosin SL indicated that the highest activator activity was obtained from the 'initial residue' (Fig. 1*i*) (see the Materials and methods section). The freeze-dried residue was extracted with 1vol. of 50mM-Tris/HCl (pH 7.5)/1mM-$MgCl_2$/1mM-dithiothreitol/5% (v/v) glycerol for 2h, centrifuged for 30min at 100000*g* and the supernatant was fractionated by collecting the protein precipitating between 40 and 60% saturation with $(NH_4)_2SO_4$ (Fig. 1*h*). All subsequent studies were made on this fraction, as activity was rapidly lost when attempts were made to fractionate it further, for example by gel filtration on Sepharose 4B. This activator preparation possessed myosin-light-chain-kinase activity.

The activator increased the Mg^{2+}-stimulated ATPase of smooth actomyosin progressively with increasing amounts (Fig. 5). It was very probably proteinaceous in nature, as it was not dialysable and was destroyed by heating for 20s at 100°C or by incubation with trypsin. Calmodulin markedly increased the activation of the actomyosin ATPase at

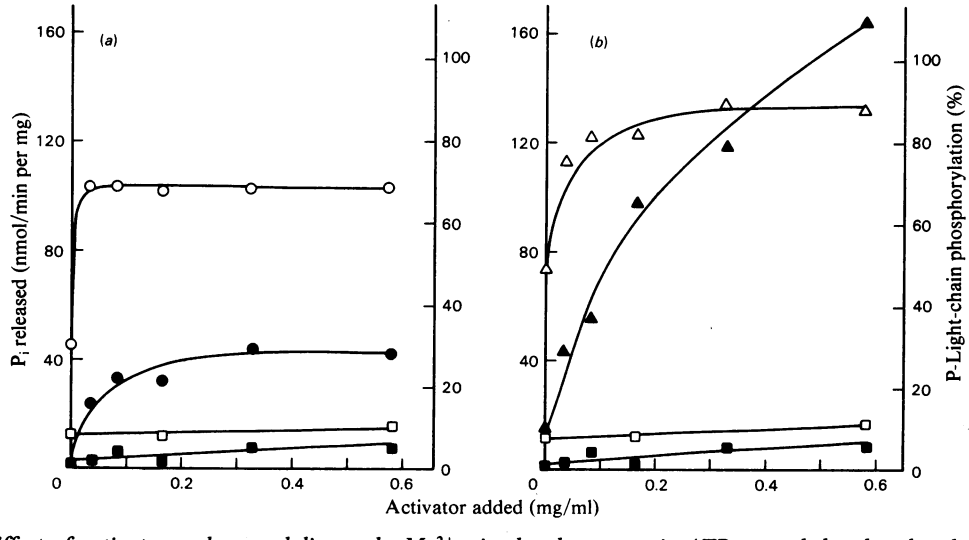

Fig. 5. *Effect of activator and calmodulin on the Mg^{2+}-stimulated actomyosin ATPase and the phosphorylation of the light chains of myosin S*

Myosin S (2mg/ml) was incubated in 50mM-Tris/HCl (pH 7.5 at 30°C)/3mM-$MgCl_2$/0.25mM-$CaCl_2$ (or 2mM-EGTA)/3.75mM-NaATP/5mM-dithiothreitol with chicken gizzard tropomyosin (0.1mg/ml) and skeletal actin (1.0mg/ml). Samples were taken from the assay mix after 4min into 2.5vol. of cold 10M-urea for the determination of light-chain phosphorylation by polyacrylamide-gel electrophoresis and phosphate release after 6min at 30°C was measured as described in the Materials and methods section. (*a*) No further additions: ○, P-light-chain phosphorylation ($CaCl_2$); □, P-light-chain phosphorylation (EGTA); ●, Mg^{2+}-ATPase ($CaCl_2$); ■, Mg^{2+}-ATPase (EGTA). (*b*) Addition of bovine brain calmodulin (25μg/ml): △, P-light-chain phosphorylation ($CaCl_2$); □, P-light-chain phosphorylation (EGTA); ▲, Mg^{2+}-ATPase ($CaCl_2$); ■, Mg^{2+}-ATPase (EGTA).

all levels of activator tested (Fig. 5). In many cases, activities of up to 250nmol of P/min per mg of myosin protein were observed, which was about a 12-fold activation of the original myosin SL. In the presence of EGTA the Mg^{2+}-stimulated ATPase activity was decreased to the same low value as that obtained with EGTA in the absence of activator (Fig. 5). There was no apparent direct correlation between ATPase activity in the presence of activator and the extent of P light-chain phosphorylation (Figs. 4 and 5). In a typical experiment illustrated in Fig. 5, in the presence of excess calmodulin, the phosphorylation was virtually complete, with very small amounts of activator (50μg/ml), and activity increased 3-fold with increasing amounts of activator, even though the light chains were fully phosphorylated.

The activator had no effect on the Ca^{2+}- and Mg^{2+}-stimulated ATPase of skeletal-muscle actomyosin. The presence of actin was necessary to obtain activation of the Mg^{2+}-ATPase of gizzard myosin SL; the Ca^{2+}-activated ATPase was not significantly affected and the EDTA/K-ATPase was slightly inhibited with increasing amounts of activator (Fig. 6).

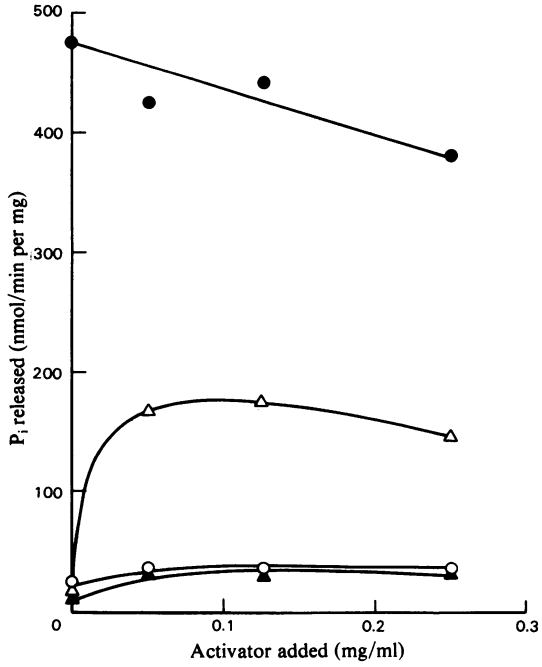

Fig. 6. *Effect of activator on the enzymic activities of gizzard myosin S*

Myosin S (1mg/ml) was incubated with various amounts of activator in 50mM-Tris/HCl (pH7.5)/5mM-dithiothreitol/5mM-ATP with calmodulin (0.01mg/ml) at 30°C and with the additions indicated: ●, 10mM-potassium EDTA + 0.6M-KCl; △, 5mM-$MgCl_2$ + rabbit skeletal actin (1mg/ml) + chicken gizzard tropomyosin (0.1mg/ml); ○, 5mM-$CaCl_2$; ▲, 5mM-$MgCl_2$ + chicken gizzard tropomyosin (0.1mg/ml).

Discussion

It is clear from the results reported that the extent of the actin activation of the Mg^{2+}-stimulated ATPase of three different preparations of smooth myosin was not proportional to the degree of phosphorylation of the P-light chain. This applied to actomyosin E and actomyosin AS, with specific activities of 10–50nmol of P/min per mg and to the myosin SL preparations which, in the presence of activator, had actomyosin ATPase specific activities of 200–250nmol of P/min per mg. The latter values are some of the highest recorded in the literature for smooth actomyosin and are comparable with those reported by Sobieszek & Small (1976). The findings reported in the present paper are in disagreement with those reporting a close correlation between P-light-chain phosphorylation and actin activation of smooth myosin ATPase (Sobeiszek & Small, 1976; Gorecka *et al.*, 1976; Chacko *et al.*, 1977), but are similar to the results of Mikawa *et al.* (1977). In all the actomyosin assays, whether carried out in the absence or presence of activator or phosphatase, the initial rate of Mg^{2+}-stimulated ATPase activity was constant. Over the time interval during which the ATPase activity remained constant the extent of P-light-chain phosphorylation changed over the range from 6 to about 90%.

In this respect it should be pointed out, however, that Persechini *et al.* (1981) have recently expressed doubts as to whether P-light-chain phosphorylation alone is adequate for activation of smooth-muscle actomyosin ATPase. It is difficult to explain why different groups of workers continue to report such contradictory results with what are apparently similar smooth-muscle myosin preparations. It could be that there exists a form of myosin, the activation of which is tightly controlled by phosphorylation of the P-light chain and which, if modified in some way, loses this property. The results with different myosin preparations, in all of which proteolytic inhibitors were used to minimize degradation, apparently successfully as judged by gel electrophoresis, argue against this explanation. In our view, different myosin preparations contain various amounts of another factor which, in the presence of calmodulin, activates the enzyme irrespective of the degree of phosphorylation of the P-light chain. The nature of the factor is as yet uncertain, and difficulties in its further purification currently exist on account of its

lability on fractionation. Since it has so far proved difficult to separate myosin light-chain kinase from the activating system, it is possible that this enzyme has a role in activation. For example, phosphorylation catalysed by the light-chain kinase may be a stage in the activation of the actomyosin ATPase. In this respect it may be significant that some degree of P-light-chain phosphorylation was apparent in all our preparations tested to date. Even if myosin light-chain kinase is involved, activation does not, in our opinion, depend on full phosphorylation of both P-light chains in the myosin molecule. The activator separates from myosin SL during its preparation with the actin-rich pellet, indicating that it may be associated with the actin filaments, as has been suggested from the work of Marston and collaborators (Marston *et al.*, 1980; Walters & Marston, 1981).

A possible means of reconciling the conflicting data in this field could be that phosphorylation of the P-light chain of smooth-muscle myosin can, under certain conditions, activate the myosin ATPase in a similar manner to that reported for skeletal myosin (Morgan *et al.*, 1976; Pemrick, 1980), and cardiac myosin (Perry *et al.*, 1978). The percentage of activation obtained on phosphorylation of myosin from striated muscles with high initial ATPase activities is relatively small (in our hands at least; cf. Pemrick, 1980) when expressed as a percentage of the control value. When expressed in absolute terms, however, it is very similar to that obtained with smooth-muscle myosin. For example, the specific activities reported for myosin after phosphorylation, with the exception of the preparation of Sobieszek & Small (1976), which we know has activator present, are in the range 50–60nmol of P/min per mg. This is approximately the increase in activity obtained after phosphorylation of skeletal or cardiac myosin. Further activation of such preparations up to specific activities of 200–250nmol of P/min per mg may be obtained when both activator and calmodulin are present. The relation of this system to leiotonin A and C described by Mikawa *et al.* (1977) is not clear, but some similarities exist. For example, calmodulin will replace leiotonin C in the latter system, but is not identical with it (Mikawa *et al.*, 1978). From the weight of evidence accumulating from several laboratories it is clear that the mechanism of the regulation of the ATPase of smooth-muscle actomyosin is complex. A simple mechanism involving phosphorylation of the P-light chains alone is no longer adequate to explain all the reported experimental facts.

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