Calcium Ion-Regulated Thin Filaments from Vascular Smooth Muscle

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(Received 20 June 1979)

Myosin and actin competition tests indicated the presence of both thin-filament and myosin-linked Ca^{2+} -regulatory systems in pig aorta and turkey gizzard smooth-muscle actomyosin. A thin-filament preparation was obtained from pig aortas. The thin filaments had no significant ATPase activity [1.1 ± 2.6 nmol/mg per min (mean $\pm$ s.d.)], but they activated skeletal-muscle myosin ATPase up to 25-fold [500 nmol/mg of myosin per min (mean $\pm$ s.d.)] in the presence of 10^{-4} M free Ca^{2+} . At 10^{-8} M- Ca^{2+} the thin filaments activated myosin ATPase activity only one-third as much. Thin-filament activation of myosin ATPase activity increased markedly in the range 10^{-6} – 10^{-5} M- Ca^{2+} and was half maximal at 2.7×10^{-6} M (p Ca^{2+} 5.6). The skeletal myosin–aorta-thin-filament mixture gave a biphasic ATPase-rate-versus-ATP-concentration curve at 10^{-8} M- Ca^{2+} similar to the curve obtained with skeletal-muscle thin filaments. Thin filaments bound up to $9.5 \mu\text{mol}$ of Ca^{2+} /g in the presence of MgATP^{2-} . In the range 0.06 – $27 \mu\text{M}$ - Ca^{2+} binding was hyperbolic with an estimated binding constant of $(0.56 \pm 0.07) \times 10^6 \text{ M}^{-1}$ (mean $\pm$ s.d.) and maximum binding of $8.0 \pm 0.8 \mu\text{mol/g}$ (mean $\pm$ s.d.). Significantly less Ca^{2+} bound in the absence of ATP. The thin filaments contained actin, tropomyosin and several other unidentified proteins. 6 M-Urea/polyacrylamide-gel electrophoresis at pH 8.3 showed proteins that behaved like troponin I and troponin C. This was confirmed by forming interspecific complexes between radio-active skeletal-muscle troponin I and troponin C and the aorta thin-filament proteins. The thin filaments contained at least $1.4 \mu\text{mol}$ of a troponin C-like protein/g and at least $1.1 \mu\text{mol}$ of a troponin I-like protein/g.

Smooth muscle, like any other muscle, contracts by means of the interaction of the contractile proteins myosin and actin. Tension and work are produced during the interaction at the expense of ATP hydrolysis at the myosin active site. The interaction is controlled by free Ca^{2+} concentrations in the range 10^{-6} – 10^{-5} M. (Ebashi & Endo, 1968; Sparrow *et al.*, 1970; Weber & Murray, 1973; Sobieszek & Bremel, 1975).

Ca^{2+} may act either by modulating the myosin or the actin-containing thin filaments (Lehman *et al.*, 1972; Lehman & Szent-Gyorgyi, 1975). Previous work on smooth-muscle regulation produced indirect evidence for a regulatory mechanism linked to the thin filaments and possibly analogous to the troponin–tropomyosin system of vertebrate striated muscle (Ebashi *et al.*, 1966; Carsten, 1971; Sparrow & Van Bockxmeer, 1972; Ito & Hotta, 1976). More recently it was discovered that smooth muscle was myosin regulated (Bremel, 1974; Sobieszek &

Bremel, 1975) and many older results were reinterpreted in terms of regulatory mechanisms involving myosin. According to current models Ca^{2+} regulates smooth-muscle myosin in two ways: by direct interaction with a regulatory light chain on the myosin molecule and by regulating a myosin light-chain kinase, which activates myosin by phosphorylating its regulatory light chain. The relative importance of the two mechanisms is uncertain (Aksoy *et al.*, 1976; Sobieszek & Small, 1977; Small & Sobieszek, 1977a,b; Chacko *et al.*, 1977; Adelstein *et al.*, 1977; Sherry *et al.*, 1978; Mrwa *et al.*, 1979). Only a few workers dissent from the view that myosin regulation is paramount (Ebashi *et al.*, 1977).

The evidence given against the existence of a thin-filament-linked regulatory system is 3-fold. First, the myosin competition test for thin-filament-linked regulation gave a negative result (Bremel, 1974; Bremel *et al.*, 1977). Secondly, smooth-muscle thin-filament preparations were not Ca^{2+} sensitive (Sobieszek & Small, 1976). Thirdly, no troponin-like proteins could be identified by SDS/polyacryl-

Abbreviation used: SDS, sodium dodecyl sulphate.

amide-gel electrophoresis of gizzard or vascular actomyosin (Sobieszek & Bremel, 1975; Driska & Hartshorne, 1975; Sparrow & Van Bockxmeer, 1972).

In our hands, however, the myosin-competition test gave a positive result, indicating the presence of thin-filament-linked regulation in both pig aorta and turkey gizzard actomyosin. We therefore decided to re-investigate the question of Ca^{2+} -regulated thin filaments in smooth muscle. We believe the work presented here refutes the evidence previously given against thin-filament regulation. This paper describes how we prepared thin filaments from pig aorta smooth muscle which activated myosin ATPase activity, which were regulated by Ca^{2+} in the range 10^{-6} – 10^{-5} M and which contained troponin-I- and troponin-C-like proteins.

Materials and Methods

Preparation of pig aorta thin filaments

Pig aorta (250 g) was minced and then homogenized in 1.5 vol. of extraction buffer [80 mM-KCl/4 mM- MgCl_2 /4 mM-EGTA/20 mM-4-morpholinepropanesulphonic acid (pH 7.0)/0.5 mM-dithiothreitol/10 mM-ATP] (Sparrow *et al.*, 1970) at 4°C. After 10 min the residue was sedimented (5 min at 14 000 g) and then homogenized in a further 1.5 vol. of extraction buffer. After 10 min the residue was again sedimented. The washed residue was homogenized in another 1.5 vol. of extraction buffer, left for 2 h to solubilize an actin-rich actomyosin and the residue was removed by a further centrifugation.

ATP (from a 100 mM pH 7.0 stock solution) was added to the supernatant to bring the concentration to 15 mM and the pH was adjusted back to 7.0. The supernatant was clarified by centrifugation for 30 min at 14 000 g and filtering through a fine nylon mesh. Solid KCl was added to a concentration of 0.5 M to optimize the dissociation of actin from myosin and the solubility of myosin. The thin filaments were then isolated by high-speed centrifugation for 3 h at 230 000 g. The pellets of thin filaments were soaked in a small volume of ATPase buffer (for composition see under 'Reconstituted actomyosin') overnight and then resuspended by using a loose-fitting Teflon/glass homogenizer. Finally, aggregated material was removed by low-speed centrifugation (1000 g). Average yield was 0.41 ± 0.08 mg of protein/g of tissue (mean $\pm$ s.d.; 11 preparations).

The other proteins

Pig aorta actomyosin was prepared by the method of Sparrow *et al.* (1970); turkey gizzard actomyosin was prepared by the method of Sobieszek & Bremel (1975). Myosin was prepared

from rabbit skeletal muscle (Perry, 1955) and from pig aorta (Sobieszek & Bremel, 1975). Pig aorta tropomyosin was made as described by Bailey (1948). Rabbit skeletal-muscle troponin I and troponin C were gifts from Dr. D. A. Mercola, A.R.C. Unit, Department of Zoology, University of Oxford, Oxford, U.K. They were radioactively labelled by incubating with a 1.5-fold molar excess of iodo[^{14}C]acetamide in 0.6 M-KCl/10 mM-Tris, pH 8.0, at 4°C for 12 h. Free radioactive label was then removed by dialysis (Marston & Weber, 1975).

Reconstituted actomyosin

ATPase measurements were made by using actomyosin reconstituted from rabbit skeletal-muscle myosin and pig aorta thin filaments. Myosin and thin filaments were mixed at the appropriate concentrations, usually 0.5 mg of myosin/ml + 1 mg of thin filaments/ml in 0.6 M-KCl/10 mM-imidazole (pH 7.0)/5 mM- MgCl_2 /10 mM-sodium azide/0.2 mM-dithiothreitol. The mixture was left for 6 h so that the two proteins could hybridize and then dialysed overnight against ATPase buffer [60 mM-KCl/10 mM-imidazole (pH 7.0 at 25°C)/5 mM- MgCl_2 /10 mM-sodium azide/0.5 mM-dithiothreitol]. Pure myosin and pure thin-filament controls were treated in the same way. For the myosin competition experiments skeletal-muscle myosin and smooth-muscle actomyosin were hybridized by this procedure.

Assay of ATPase activity

The ATPase activity of myosin, thin filaments and reconstituted actomyosin was measured at 25°C in ATPase buffer. The reaction was started by adding MgATP^{2-} to 2 mM and terminated after 0, 1, 2 or 3 min with an equal volume of 5% trichloroacetic acid. P_i released was measured by the Taussky & Schorr (1953) method; it was linear with time for at least 5 min and so the rate was calculated by a least-squares fit of the P_i -versus-time data.

Some measurements of ATPase activity were made over a range of MgATP^{2-} concentrations from 10^{-6} to 10^{-3} M. In these experiments 2.5 mM-phosphocreatine and 1 mg of creatine kinase/ml were used to regenerate ATP from ADP. The reaction was terminated with 5 mM-*p*-hydroxymercuribenzoate. Protein was precipitated by ZnSO_4 and subsequently removed by $\text{Ba}(\text{OH})_2$ (Somogyi, 1945). The creatine released was assayed by the Eggleton *et al.* (1943) method.

Maintenance of Ca^{2+} concentration

Ca^{2+} concentrations were maintained in the range 10^{-8} to 10^{-4} M by use of CaEGTA buffers (Portzehl *et al.*, 1964). In general the CaCl_2 concentration was constant at 102 μM , this value being standardized by atomic-absorption spectroscopy. Ca^{2+} concentration was then varied by adding 0–2 mM-EGTA.

The Ca^{2+} concentration was calculated by using the ion-binding computer programme of Perrin & Sayce (1967) with the stability constants listed by White & Thorson (1971). The apparent CaEGTA -binding constant under our incubation conditions was $3.0 \times 10^6 \text{ M}^{-1}$.

Ca^{2+} binding to thin filaments

Ca^{2+} binding was measured by the double-labelling technique of Kendrick-Jones *et al.* (1970). Thin filaments (1 mg) were incubated with $102 \mu\text{M}$ - $^{45}\text{CaCl}_2$ and 5 mM- ^3H glucose as an inert volume marker. The filaments, together with their bound Ca^{2+} , were separated by centrifugation for 1½ h at 180 000 g; 70–80% of the protein sedimented under these conditions. Pellets were resuspended in 10 mM-Tris, pH 8.5. The ^3H and ^{45}Ca radioactivity in the pellets and an aliquot of the supernatant were measured, from which the amount of bound Ca^{2+} was calculated.

Gel electrophoresis

Polyacrylamide (10%, w/v) slab gels were run with 0.1% SDS, 100 mM-sodium phosphate, pH 7.0, for 4 h at 40 V and stained in Coomassie Blue or Fast Green FCF. For quantitative estimates 6% gels were run and stained in Coomassie Blue. The separated protein bands were cut out, eluted from the gel in 25% (v/v) pyridine and their A_{605} values were measured (Fenner *et al.*, 1975). The assay was calibrated by running pure protein standards of known concentration with thin-filament preparations on the same slab gel.

Polyacrylamide (8%, w/v)/6 M-urea/20 mM-Tris/glycine (pH 8.3) slab gels were run for 1½ h at 200 V and stained in Fast Green FCF (Perry *et al.*,

1972). Some urea gels were run with radioactively labelled troponin I and troponin C. To assay the position and amount of radioactivity the stained gels were cut into ten equal sections, which were ground up, dissolved by heating in 30% H_2O_2 (Young & Fulhorst, 1965) and then assayed by liquid-scintillation counting.

Results

Smooth-muscle actomyosin has both actin- and myosin-linked Ca^{2+} regulation

We used the tests devised by Lehman & Szent-Gyorgi (1975) to test for thin-filament-linked regulation in pig aorta and turkey gizzard smooth muscle. The tests are indirect, but they have the advantage that they can probe for the Ca^{2+} -regulating potential of thin filaments in crude preparations, which are obtained with the minimum of manipulation. This gives us the best chance of detecting a regulatory system, which is easily lost or damaged during preparation.

Ca^{2+} -regulated smooth-muscle actomyosin and skeletal-muscle myosin were mixed in 0.6 M-KCl/10 mM-imidazole (pH 7.0)/5 mM- MgCl_2 and then dialysed against ATPase buffer. The ATPase activity of the mixture was measured in 10^{-4} M- and 10^{-8} M- Ca^{2+} and compared with the ATPase activities of the smooth-muscle actomyosin and skeletal-muscle myosin measured separately under the same conditions (Table 1).

With both aorta and gizzard actomyosin the ATPase activity of the mixture was substantially greater than the sum of its component ATPases due to myosin and actomyosin; thus the skeletal-muscle

Table 1. *Myosin and actin competition tests on smooth-muscle actomyosins*

Means $\pm$ S.D. are given for Ca^{2+} sensitivities determined in individual preparations, for the number of preparations in parentheses.

	Pig aorta			Turkey gizzard		
	ATPase activity (nmol/ml per min)		Ca^{2+} sensitivity (%)†	ATPase activity (nmol/ml per min)		Ca^{2+} sensitivity (%)
	10^{-4} M- Ca^{2+}	10^{-8} M- Ca^{2+}		10^{-4} M- Ca^{2+}	10^{-8} M- Ca^{2+}	
(A) Actomyosin (1 mg/ml)	26.0	6.5	68 ± 14 (13)	23.0	4.3	81 ± 22 (6)
(B) Actomyosin (1 mg/ml) + rabbit fast myosin (1 mg/ml)*	154	66	56 ± 11 (7)	171	60	54 ± 20 (6)
(C) (B)–(A)	128	60	57 ± 12 (7)	148	55	46 ± 19 (6)
(D) Actomyosin (1 mg/ml) + + tropomyosin–actin (1 mg/ml)	66	34	58 ± 18 (6)	51	5	88 ± 6 (4)

* ATPase activity due to fast muscle myosin (15 nmol/mg of myosin/min) subtracted

† Ca^{2+} sensitivity = $\frac{(\text{ATPase at } 10^{-4} \text{ M-}\text{Ca}^{2+}) - (\text{ATPase at } 10^{-8} \text{ M-}\text{Ca}^{2+})}{(\text{ATPase of } 10^{-4} \text{ M-}\text{Ca}^{2+})} \times 100$

myosin and smooth-muscle actomyosin were interacting. The most probable explanation is that the skeletal-muscle myosin had hybridized with the thin filaments in the smooth-muscle actomyosin; the hybrid actomyosin would then have a high ATPase activity characteristic of actin-activated skeletal-muscle myosin.

The excess ATPase activity arising from this interaction (Table 1; C) was Ca^{2+} -sensitive (42–61% in aorta, 26–67% in gizzard). Since skeletal-muscle myosin as normally prepared is not Ca^{2+} -regulated, we inferred that the smooth-muscle thin filaments were themselves Ca^{2+} -regulated.

Myosin-linked regulation was tested by an actin-competition test that is the converse of the myosin-competition test. Smooth-muscle actomyosin (1 mg/ml) was mixed in the high-salt solution with smooth-muscle tropomyosin/rabbit F-actin complex (1 mg/ml) which has no intrinsic ATPase activity and, lacking troponin, is not Ca^{2+} -sensitive when tested with rabbit skeletal myosin. After 6 h the mixture was dialysed against the buffer used for ATPase assay. The ATPase activity of the mixture was assayed at 10^{-4}M - and 10^{-8}M - Ca^{2+} .

If the unregulated tropomyosin/actin displaces the smooth-muscle thin filaments from an unregulated smooth-muscle myosin, the mixture should have little or no Ca^{2+} sensitivity, but if the smooth-muscle myosin is Ca^{2+} -regulated, Ca^{2+} sensitivity will be retained in the mixture. We found that the ATPase activity of the mixture was greater than that of its components and was still Ca^{2+} -sensitive (39–83% in pig aorta, 79–92% in turkey gizzard), in agreement with data published previously (Bremel, 1974; Litten *et al.*, 1979; Table 1). From this result we inferred that the myosin of gizzard and pig aorta actomyosin is Ca^{2+} -regulated.

Previous work using the myosin-competition principle has either shown no evidence for thin-filament regulation (Bremel, 1974; with turkey gizzard) or given an ambiguous result (Litten *et al.*, 1979; with bovine aorta). However, we obtained a very consistent positive result. We therefore sought to confirm the predictions of the competition test by isolating a native thin-filament preparation from smooth muscle.

Smooth-muscle thin filaments activate skeletal-muscle myosin

The aorta thin-filament preparation was a turbid solution that was not viscous, even at 8 mg/ml. Electron micrographs show that the preparation consists of rather short filaments [length was $200 \pm 80\text{ nm}$ (mean $\pm$ s.d.) (equivalent to 70 actin monomers)]. Filaments had a typical actin structure and could be decorated with rabbit skeletal-muscle S-1.

Aorta thin filaments had a negligible ATPase

Fig. 1. Activation of skeletal-muscle myosin ATPase activity by aorta thin filaments

Skeletal-muscle myosin (0.5 mg/ml), aorta thin filaments (0–5 mg/ml), 2 mM-MgATP²⁻, ATPase buffer (pH 7.0 at 25°C) plus 10^{-4}M - Ca^{2+} (●) or 10^{-8}M - Ca^{2+} (○) were present.

activity by themselves [$1.1 \pm 2.6\text{ nmol/mg}$ of thin filaments per min (mean $\pm$ s.d.; 12 assays)], but they did activate rabbit skeletal-muscle myosin ATPase at 10^{-4}M - Ca^{2+} (Fig. 1). The standard synthetic actomyosin containing 1 mg of thin filaments/ml and 0.5 mg of skeletal-muscle myosin/ml had an ATPase activity of $119 \pm 11\text{ nmol/mg}$ of myosin per min (mean $\pm$ s.d.; 7 preparations) compared with $15 \pm 5\text{ nmol/mg}$ of myosin per min (mean $\pm$ s.d.) for myosin alone. Gizzard thin filaments gave a similar activation.

In the range of thin-filaments/myosin ratios studied the activation of ATPase activity increased almost linearly with the relative amount of thin filaments present (Fig. 1). The fastest rate we obtained (500 nmol/mg of myosin per min at a 10:1 weight ratio) was equivalent to a myosin turnover rate of 1.9 s^{-1} per myosin head. Hence the maximum activity obtained at infinite thin-filament concentration is expected to be at least twice this value. The activation of skeletal myosin ATPase activity by aorta smooth-muscle thin filaments is therefore comparable with activation by skeletal-muscle thin filaments (maximum $2.5\text{--}6\text{ s}^{-1}$; Pemrick & Weber, 1976).

Activation by smooth-muscle thin filaments is Ca^{2+} -regulated

The myosin-competition test predicts that smooth-muscle thin filaments should be Ca^{2+} -regulated (Table 1) and this was found to be so. When the aorta thin filaments are prepared by our procedure they activate rabbit skeletal-muscle myosin activity up to four times more at 10^{-4}M - Ca^{2+} than at 10^{-8}M - Ca^{2+} (Fig. 1). The standard reconstituted

actomyosin (0.5 mg of myosin/ml + 1.0 mg of thin filaments/ml) gave an activation (i.e. total ATPase — myosin ATPase activity) of 34 ± 11 nmol/mg of myosin per min at 10^{-8} M- Ca^{2+} (mean $\pm$ s.d.) compared with 104 ± 11 nmol/mg of myosin per min at 10^{-4} M-calcium (mean $\pm$ s.d.; eight preparations), equivalent to a Ca^{2+} sensitivity of 70%.

When the Ca^{2+} concentration was varied between 10^{-8} M and 10^{-4} M it was found that the increase in activation of ATPase activity occurred over a narrow range of Ca^{2+} concentrations at 2.5–3 μM . (p Ca^{2+} 5.5 to 5.6). Fig. 2(a) shows the dependence of activation on log(Ca^{2+} concentration), and Fig. 2(b) shows the activation normalized and plotted directly against Ca^{2+} concentration. The curve is sigmoid; the increase in activation changed from 10 to 90% over a 10-fold or less change in Ca^{2+} concentration.

Fig. 2. Dependence of the activation of myosin ATPase activity by aorta thin filaments on Ca^{2+} concentration. Activation = total ATPase activity — myosin ATPase activity. Skeletal-muscle myosin (0.5 mg/ml), aorta thin filaments (1 mg/ml), 2 mM-MgATP²⁻, ATPase buffer (pH 7.0 at 25°C) and 10^{-8} – 10^{-4} M- Ca^{2+} were present. (a) shows the dependence of activation of ATPase activity on log (Ca^{2+} concentration) (results are for one experiment). (b) shows the dependence of activation of ATPase activity on Ca^{2+} concentration. The data were normalized. Thus 0% = ATPase activity at 10^{-8} M- Ca^{2+} , 100% = ATPase activity at 10^{-4} M- Ca^{2+} . Points are means $\pm$ s.d. for five experiments with different thin-filament preparations.

This indicates some form of co-operative interaction between the sites where Ca^{2+} binds and the myosin-binding site of the actin where its effect is manifested (Weber & Bremel, 1971; Bremel & Weber, 1972; Weber & Murray, 1973). Pig aorta actomyosin gave a similar dependence of ATPase activity on Ca^{2+} concentration with half-maximal change of ATPase activity also at about 2.5 μM .

The activation of skeletal-muscle myosin by aorta thin filaments was regulated by Ca^{2+} at all thin-filament/myosin ratios tested (Fig. 1). Ca^{2+} sensitivity was dependent on MgATP²⁻ concentration (Fig. 3). Below 6 μM -ATP the aorta thin filaments were unregulated; with increasing ATP concentrations above 10^{-5} M the ATPase activity at 10^{-4} M- Ca^{2+} continued to increase to a maximum, but ATPase activity at 10^{-8} M- Ca^{2+} actually decreased before levelling off at about 10^{-4} M-ATP. The biphasic response at 10^{-8} M- Ca^{2+} , which was consistently observed, resembles that obtained with skeletal-muscle actomyosin (Weber & Bremel, 1971; Bremel & Weber, 1972) rather than smooth-muscle actomyosin, where, in common with other workers (Bremel, 1974; Hartshorne *et al.*, 1977; Ebashi *et al.*, 1977) we obtained an almost linear relationship between ATPase activity and log (ATP concentration) at both 10^{-8} M- and 10^{-4} M- Ca^{2+} .

Ca^{2+} binding

We measured Ca^{2+} binding to aorta thin filaments under the same conditions as we measured ATPase activity; 2 mM-MgATP²⁻ in ATPase buffer at 25°C.

Fig. 3. Dependence of the activation of myosin ATPase activity by aorta thin filaments on MgATP²⁻ concentration.

Activation = total ATPase activity — myosin ATPase activity. Skeletal-muscle myosin (0.5 mg/ml), aorta thin filaments (1 mg/ml), 10^{-6} – 10^{-3} M-MgATP²⁻, 2.5 mM-phosphocreatine, creatine kinase (1 mg/ml), ATPase buffer (pH 7.0 at 25°C) and 10^{-4} M- Ca^{2+} (●) or 10^{-8} M- Ca^{2+} (○) were present.

Fig. 4. Ca^{2+} binding to aorta thin filaments

(a) shows the dependence of Ca^{2+} binding on log (Ca^{2+} concentration). Symbols: ●, 2 mM-MgATP²⁻; ○, no ATP. ATPase buffer (pH 7.0) at 25°C was present. Points are means $\pm$ 1 s.d. for four experiments with two different preparations. The solid line is the best fit of data in the range of values of log (Ca^{2+} concentration) from -7.1 to -4.5 to an equation of simple binding. The curve has the parameters: $B_m = 7.8 \mu\text{mol/g}$ of thin filaments; $K_b = 0.59 \times 10^6 \text{ M}^{-1}$. (b) shows data from (a) replotted as a Scatchard plot.

The bound Ca^{2+} increased with increasing Ca^{2+} concentration in a regular manner, reaching $9.5 \mu\text{mol/g}$ of thin filaments at $70 \mu\text{M}$ - Ca^{2+} (Fig. 4). With the exception of the highest Ca^{2+} concentration, the data closely approximated a simple binding curve (solid line on Fig. 4) as indicated by the linearity of the Scatchard plot (Fig. 4b). The data in the range 0.06 to $27 \mu\text{M}$ were therefore analysed by a least-squares fit to the equation:

$$\text{Bound Ca}^{2+} = \frac{\text{maximum bound Ca}^{2+} (B_m) \times [\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + \left(\frac{1}{\text{binding constant } (K_b)} \right)}$$

(Wilkinson, 1961). The mean ($\pm$ s.d.) for four estimates of B_m was $8.0 \pm 0.8 \mu\text{mol/g}$ and the mean ($\pm$ s.d.) binding constant was $(0.56 \pm 0.07) \times 10^6 \text{ M}^{-1}$. The data were insufficient to justify fitting to an equation for more than one class of site. However, the presence of low-affinity Ca^{2+} binding in addition to the observed high-affinity binding may distort the curve slightly in the direction of giving apparently higher B_m and lower K_b values than the true values. Pure aorta smooth-muscle myosin bound up to $1.8 \mu\text{mol}$ of Ca^{2+}/g under the conditions used for measuring thin-filament Ca^{2+} binding. Since contaminating myosin makes up less than 20% of the mass of the thin-filament preparations a maximum of $0.36 \mu\text{mol}$ of bound Ca^{2+}/g of thin filaments could be due to the myosin contaminant. The maximum Ca^{2+} binding by aorta thin filaments was quite similar to the amount of Ca^{2+} bound by reconstituted skeletal-muscle thin filaments under the same conditions (6 – $9 \mu\text{mol/g}$).

When Ca^{2+} binding to aorta thin filaments was measured in the absence of MgATP^{2-} , significantly

Fig. 5. Densitometer scan of 10% polyacrylamide/0.1% SDS-gel electrophoresis of aorta thin filaments. The gel was stained with Fast Green FCF and scanned by a Vitatron densitometer; $100 \mu\text{g}$ of aorta thin filaments was used. The numbers in the Figure give approximate molecular weights of the peaks.

less Ca^{2+} was bound and the Scatchard plot was strongly curved.

Protein components of aorta thin filaments

We analysed the aorta thin-filament preparations by gel electrophoresis. Fig. 5 shows a typical densitometer scan of aorta thin filaments electrophoresed in 10% polyacrylamide/0.1% SDS. The peaks labelled with approximate molecular weights were consistent features of the preparation. Gels (6%) revealed an additional minor component with a chain mol.wt. greater than 200 000. The major components, with mol.wts. 42 000 and 35 000, co-

migrate with actin and tropomyosin respectively. Quantitative measurement of the stained bands gave a ratio for actin/tropomyosin of 0.79 ± 0.08 (mean $\pm$ S.D., $n=5$) by weight or 0.66 ± 0.07 (mean $\pm$ S.D.) by molarity. There was therefore an apparent excess of tropomyosin compared with skeletal-muscle thin filaments (molar ratio 7:1) (Bremel & Weber, 1972; Potter, 1975). This may represent a genuinely higher tropomyosin content in aorta thin filaments or the presence of an additional protein or proteins with the same mobility as tropomyosin.

The mol.wt.-195000 protein is a contaminant of myosin heavy chain, present to a small extent in all preparations. The actin/myosin-heavy-chain ratio was found to be 10 ± 2 (mean $\pm$ S.D.; $n=5$) on a molar basis. Since thin-filament preparations had no intrinsic ATPase activity this myosin was inactive. The other components of the thin-filament preparations have not been positively identified; the 20000- and 17000-mol.wt. proteins may be contaminants from myosin light chains (Kendrick-Jones, 1973), although the quantity of 20000-mol.wt. protein appears to be too much to be accounted for by myosin light chain alone.

Electrophoresis of aorta thin filaments in 8% polyacrylamide/6M-urea/20 mM-Tris/glycine (pH 8.3) is shown in Fig. 6. The main peaks a, c and d have not been positively identified although peak c co-migrated with tropomyosin and peak d co-mi-

Fig. 6. Densitometer scan of 8% polyacrylamide/6M-urea/20 mM-Tris/glycine (pH 8.3)-gel electrophoresis of aorta thin filaments

Gels were stained with Fast Green FCF and scanned by a Vitatron densitometer; 200 μ g of aorta thin filaments was used. Upper trace, electrophoresis in the presence of 10 mM-EGTA; lower trace, electrophoresis in the presence of 10 mM- CaCl_2 .

grated with a myosin light chain. The protein labelled peak e ran with a high mobility in the presence of 10 mM-EGTA and had a mobility identical with skeletal-muscle troponin C. Protein corresponding to peak e was absent when the gel was run in the presence of 10 mM- CaCl_2 , but a new peak (b) appeared, which had a mobility only one-third that of the protein corresponding to peak e. This pattern of results is closely analogous to that observed with skeletal-muscle thin filaments (Perry *et al.*, 1972; Head & Perry, 1974). Skeletal-muscle troponin I and troponin C form a complex in the presence of Ca^{2+} that is stable in 6M-urea and has a mobility intermediate between that of troponin C and troponin I, which does not migrate into the gel. The skeletal-muscle complex is not formed in the absence of Ca^{2+} so the peak due to free troponin C is then visible.

Our observations on aorta thin filaments therefore suggested that our preparations contained troponin I- and troponin C-like proteins. We tested this hypothesis by running mixtures of aorta thin filaments and radioactively labelled skeletal-muscle troponin C or troponin I in 6M-urea in the presence of 10 mM- CaCl_2 (Fig. 7). If the aorta smooth-muscle thin filaments contain functional troponin I- and troponin C-like proteins we may expect them to form interspecific complexes of intermediate mobility with the radioactive troponin C and troponin I in the presence of Ca^{2+} (Grand *et al.*, 1979), and they do. Pure skeletal-muscle troponin I does not migrate into the gel (Fig. 7a), but in the presence of a 10-fold excess (by weight) of aorta thin filaments, $24 \pm 8\%$ (mean $\pm$ S.D.; 15 determinations) of the skeletal-muscle troponin I was found to migrate with a mobility of 0.3 to 0.5 (Fig. 7c) (mobility is expressed relative to the most mobile band, troponin C). The quantity of skeletal-muscle troponin I in the intermediate mobility complex increased with increasing troponin I/thin-filaments ratio (Fig. 7e); the highest value (obtained at 1:2 by weight) was 1.45 μ mol of skeletal-muscle troponin I/g of thin filaments.

Pure skeletal-muscle troponin C had a mobility of 1.0 (by definition) (Fig. 7b), but when mixed with an excess of aorta thin filaments, $19 \pm 9\%$ (mean $\pm$ S.D.; 14 determinations) of the troponin C migrated with a mobility of 0.8–0.9 (Fig. 7d). Up to 1.1 μ mol of troponin C was observed to be incorporated into the intermediate mobility complex of thin filaments (Fig. 7f). Intermediate mobility complexes were not observed if Ca^{2+} was not present in the gel medium.

When radioactive troponin C and troponin I were mixed with actin, tropomyosin and myosin in a weight ratio of 1:1.2:0.4, which corresponds to their relative abundance in thin-filament preparations (Fig. 5), very little of the radioactivity migrated at intermediate mobility (2.4% of troponin I, <3% of troponin C). This result rules out the possibility

Fig. 7. 8%-Polyacrylamide/6 M-urea (pH 8.3)-gel electrophoresis of radioactively labelled troponin I and troponin C with and without aorta thin filaments

Gels were run in 10 mM- CaCl_2 . Stained gels were cut into ten equal parts and their radioactivity was determined. (a)–(d) show the distribution of radioactivity; a relative mobility of 1.0 corresponds to the mobility of skeletal-muscle troponin C in 10 mM- CaCl_2 . (a) 20 μg of radioactive skeletal-muscle troponin I; (b) 20 μg of radioactive skeletal-muscle troponin C; (c) 20 μg of radioactive troponin I + 200 μg of aorta thin filaments; (d) 20 μg of radioactive troponin C + 200 μg of aorta thin filaments. (e) and (f) show the quantity of radioactive troponin I or troponin C respectively that had altered electrophoretic mobility in the presence of 200 μg of aorta thin filaments plotted against the added amount of radioactive troponin I or troponin C. (e) shows radioactive troponin I migrating with relative mobility 0.3–0.5 in the presence of aorta thin filaments. (f) shows radioactive troponin C migrating with relative mobility 0.8–0.9 in the presence of aorta thin filaments.

that the intermediate mobility complexes were formed by non-specific association with the major thin-filament proteins. Therefore we conclude that aorta thin filaments contain at least 1.45 μmol of troponin C-like protein/g and at least 1.1 μmol of a troponin I-like protein/g.

Discussion

The myosin- and actin-competition tests (Table 1) have demonstrated the existence of a potentially functional actin-linked Ca^{2+} -regulatory system in arterial and gizzard smooth muscle and have confirmed the already well-documented presence of a myosin-linked regulatory system. The finding of actin-linked regulation seemed to be at variance with published work on smooth-muscle thin filaments (Bremel *et al.*, 1977), so we have investigated this hypothesis further.

We did this by isolating native thin filaments from smooth muscle and testing their ability to interact with vertebrate skeletal-muscle myosin. We used skeletal myosin because it is well characterized. In particular it is known that the enzymic mechanism of actin-activated ATP hydrolysis is the same as that of smooth muscle (Marston & Taylor, 1978) and that, as commonly prepared, it is not regulated by Ca^{2+} ; therefore any Ca^{2+} -sensitive ATPase activity observed in a reconstituted skeletal-muscle myosin–smooth-muscle thin-filament complex may be ascribed to regulation of the thin filaments.

The native thin filaments from pig aorta smooth muscle activate skeletal-muscle myosin almost as much as do skeletal-muscle thin filaments (Fig. 1). The activation is Ca^{2+} -regulated (Figs. 1 and 2) and the thin filaments bind substantial amounts of Ca^{2+} at high affinity (Fig. 4). Turkey gizzard thin filaments activated myosin ATPase activity, but were not Ca^{2+} -regulated, in agreement with the finding of Sobieszek & Small (1976). Since the myosin-competition test for thin-filament regulation gave equally positive results in gizzard and aorta actomyosin (Table 1), we think it is likely that the failure to observe Ca^{2+} sensitivity in gizzard thin filaments was due to an inadequate preparation method.

How are the aorta thin filaments regulated? The first question we asked was whether the regulatory factors were Ca^{2+} -dependent activators of normally inactive thin filaments or inhibitors of normally active thin filaments that were de-repressed by Ca^{2+} . The ideal experiment would be to determine the effect on ATPase activity of removing or inactivating the regulatory factors (desensitization). Unfortunately the thin filaments proved to be resistant to attempts at desensitization by digestion with trypsin, α -chymotrypsin or subtilisin BNP [30 min at 0°C with 1 μg of enzyme/g of thin filaments (Ebashi, 1963)] or by washing at low salt concentration (Schaub *et al.*, 1967). Nevertheless an answer to the question may be obtained by comparing the ATPase activities obtained with thin-filament preparations having different Ca^{2+} sensitivities (Fig. 8). We found little correlation between the amount of activation of myosin ATPase at 10^{-4} M- Ca^{2+} and the Ca^{2+} sensi-

Fig. 8. Relationship between activation of myosin ATPase activity by aorta thin filaments and Ca^{2+} sensitivity. The data are from nine thin-filament preparations assayed with 0.5 mg of skeletal-muscle myosin/ml, 1 mg of aorta thin filaments/ml, 2 mM-MgATP²⁻, ATPase buffer (pH 7.0 at 25°C) and 10^{-4} or 10^{-8} M- CaCl_2 . Ca^{2+} sensitivity is defined in Table 1. Symbols: O, activation at 10^{-8} M- Ca^{2+} (correlation coefficient -0.98); ●, activation at 10^{-4} M- Ca^{2+} (correlation coefficient -0.55). The broken lines represent the result expected from a depressor-type regulatory mechanism.

tivity, but at 10^{-8} M- Ca^{2+} there was a significant negative correlation (correlation coefficient -0.98), i.e. high Ca^{2+} sensitivity (or good regulation) correlated with maximal inhibition of activation at low Ca^{2+} concentration. This observation is compatible with aorta thin filaments that are intrinsically active being inhibited by regulatory factors and depressed by Ca^{2+} , analogous to the familiar troponin-tropomyosin system of skeletal muscle (Ebashi & Endo, 1968). A Ca^{2+} -dependent activator mechanism would have given the opposite result (Ebashi *et al.*, 1977).

A loss of Ca^{2+} sensitivity at low MgATP²⁻ concentration and a biphasic rate-versus-log [ATP] curve was observed with reconstituted actomyosin containing aorta thin filaments and skeletal-muscle myosin (Fig. 3). The curve is similar to curves obtained in skeletal-muscle actomyosin, which have been interpreted as being caused by co-operative interactions between the myosin-actin-binding site, tropomyosin and troponin (Weber & Bremel, 1971;

Bremel & Weber, 1972). Therefore the observation of a biphasic curve with aorta thin filaments suggests that they have a regulatory system resembling troponin-tropomyosin of skeletal muscle. Smooth-muscle actomyosin does not give a biphasic rate-versus-log [ATP] curve at low Ca^{2+} concentration (Bremel *et al.*, 1977; Ebashi *et al.*, 1977). This is believed to be a consequence of the myosin-linked regulation system, which behaves differently from the thin-filament-linked system (Bremel, 1974; Bremel *et al.*, 1977). If both myosin- and thin-filament-linked systems operate in smooth muscle it is necessary to propose that the inhibitory effect of the former overrides loss of regulation in the thin filaments at low ATP concentrations.

Since the aorta thin filaments functioned in a manner similar to the troponin-tropomyosin-regulated thin filaments of skeletal muscle we searched for troponin components in our thin-filament preparations. Aorta thin-filament preparations contained actin, tropomyosin and a number of unidentified protein components that could be regulatory proteins (Fig. 5). These included proteins with mol.wts. 26000 and 20000, which may correspond to smooth-muscle troponin I-like protein and troponin C-like protein (mol.wts. 25000 and 18500 respectively according to Grand *et al.*, 1977). Gel electrophoresis in 6M-urea at pH 8.3 demonstrated the presence of a protein with the same characteristically high mobility as skeletal-muscle troponin C in 10 mM-EGTA, which ran at an intermediate mobility in 10 mM- CaCl_2 (Fig. 6), analogous to the urea-stable troponin I-troponin C complex of skeletal muscle (Head & Perry, 1974). Interspecific complexes could be formed between skeletal-muscle troponin C and an aorta troponin I-like protein and between skeletal-muscle troponin I and an aorta troponin C-like protein (Fig. 7), thereby establishing a functional homology between skeletal-muscle and smooth-muscle proteins.

It is known that whole homogenates of smooth muscle contain a substantial amount of calmodulin (also known as Ca^{2+} -dependent regulator and modulator protein) (Grand *et al.*, 1977, 1979; Dabrowska *et al.*, 1978) and in some of the experiments described above troponin C is indistinguishable from calmodulin (Vanaman *et al.*, 1976; Grand *et al.*, 1979). However, we believe that the balance of evidence favours the aorta thin-filament protein being a troponin C rather than calmodulin for two reasons. First, the thin filaments are prepared by washing and sedimentation in the presence of 4 mM-EGTA, conditions that are optimal for releasing bound calmodulin into the supernatant (Vanaman *et al.*, 1976). Secondly, we observed a complex between skeletal-muscle troponin I and the thin-filament troponin C-like protein in 6M-urea (Fig. 6c), yet the calmodulin-troponin I complex is

reported to be unstable in greater than 5 M-urea (Grand *et al.*, 1979).

The estimated quantity of aorta troponin C- and troponin I-like proteins [at least $1.5 \mu\text{mol/g}$ of thin filaments (Fig. 7)] approaches the quantity of troponin in skeletal-muscle thin filaments [$2.2 \mu\text{mol/g}$, on the basis of 1 troponin and 1 tropomyosin molecule/7 molecules of actin (Bremel & Weber, 1972; Potter, 1975)]. The quantity of Ca^{2+} bound at high affinity [$8.0 \mu\text{mol/g}$ of aorta thin filaments (Fig. 4)] is also similar to that bound by skeletal-muscle thin filaments [$8.8 \mu\text{mol/g}$ on the basis of 4 Ca^{2+} -binding sites/troponin molecule (Potter & Gergely, 1974)] and the affinity is compatible with the Ca^{2+} concentration required to activate aorta thin filaments (Figs. 2 and 4).

Aorta thin filaments therefore contain the necessary proteins for regulation by a troponin-tropomyosin system and exhibit the functional characteristics (regulation by de-repression, cooperativity and Ca^{2+} binding) that are expected from such a system. We have demonstrated the potential, but it remains to be shown that smooth-muscle troponin-tropomyosin functions *in vivo*.

The properties of our aorta thin filaments differ quite markedly from the system described by Ebashi and his co-workers in a series of preliminary communications (Ebashi *et al.*, 1975, 1977; Mikawa *et al.*, 1977a,b, 1978; Hirata *et al.*, 1977). They found that smooth-muscle actomyosin was not active unless tropomyosin, a protein of mol.wt. 80000 called leiotonin, which is associated with the thin filaments and, perhaps, a protein of mol.wt. 20000 were present. In the presence of small amounts of leiotonin the actomyosin was active and Ca^{2+} -regulated. Some properties of leiotonin resemble myosin light-chain kinase (Aksoy *et al.*, 1976; Sobieszek & Small, 1976; Chacko *et al.*, 1977), but this is apparently not the case since leiotonin has no kinase activity. It is difficult to see any parallel between leiotonin and the thin-filament regulation we have observed. It is conceivable that leiotonin was lost or permanently 'switched on' in our preparations.

Smooth muscle is the first vertebrate muscle in which Ca^{2+} regulation of both thick and thin filaments has been clearly demonstrated, although there is some evidence that fast skeletal muscle may also be dual regulated (Lehman, 1978). In invertebrate muscles dual regulation is almost universal (Lehman & Szent-Gyorgyi, 1975). Clearly dual regulation has physiological advantages. Indeed it has been shown that two systems can act synergistically to improve the response to small changes in Ca^{2+} concentration (Lehman & Szent-Gyorgyi, 1975). Accurate control of smooth-muscle tone *in vivo* is extremely important in homeostatic mechanisms, such as the control of blood pressure. It is hoped that further

study of vascular smooth-muscle regulation will reveal how the multiple forms of Ca^{2+} -regulation of contraction work together to achieve this.

We thank Dr. W. Lehman for advice on the proper use of competition tests, Dr. Roger Craig for making electron micrographs of our thin filaments and Simpson's Ltd., Hazelgrove, Stockport, Cheshire, U.K., for the supply of pig aortas.

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