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Phosphorylation of the calcium ion-regulated thin filaments from vascular smooth muscle

A new regulatory mechanism?

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The thin filaments of vascular smooth muscle (pig aorta) contain a Ca^{2+}-sensitive regulatory system that resembles troponin–tropomyosin [Marston, Trevett & Walters (1980) *Biochem. J.* **185**, 355–365]. Our thin-filament preparations also contain enzymes that phosphorylate and dephosphorylate a specific protein. Initial rate of phosphorylation was 0.42 ± 0.10 (95% confidence limits) μmol of P_i/min per g of thin filaments; half-maximal incorporation was obtained in $4\frac{1}{2}$min, and a maximum of 1.8 ± 0.1 μmol of P_i/g of thin filaments was incorporated after 40min (conditions: 1mM-MgATP, 60mM-KCl, 10mM-imidazole, pH 7.0, 5mM-$MgCl_2$, 10mM-NaN_3, 0.5mM-dithiothreitol, 0.1mM-$CaCl_2$, 25°C). On gel electrophoresis in polyacrylamide (4–30% gradient)/0.25% sodium dodecyl sulphate gel over 75% of protein-bound phosphate was in a single protein of mol.wt. 21000. On electrophoresis in polyacrylamide (8%)/6M-urea (pH 8.6) gel the phosphoprotein remained at the origin. Phosphorylation was associated with an increase in the concentration of high-affinity ($K \simeq 10^6 M^{-1}$) Ca^{2+}-binding sites from 0.8–1.5 to 6.3 μmol of Ca^{2+}/g of thin filaments. Phosphorylation also changed the regulatory properties of the skeletal-muscle myosin–aorta thin-filament MgATPase; maximum activity was unaltered, but the phosphorylated thin filaments required only 0.36 μM-Ca^{2+} for half-activation compared with 2.7 μM-Ca^{2+} for unphosphorylated thin filaments. The possible regulatory role of thin-filament phosphorylation is discussed.

Contraction of vascular smooth muscle is produced by the interaction of the contractile proteins, actin and myosin (Murphy, 1979). Tension and work are produced by this interaction at the expense of MgATP hydrolysis at the myosin active site (Paul *et al.*, 1976; Marston & Taylor, 1980). The degree of actin–myosin interaction is precisely controlled by the concentration of Ca^{2+} in the sarcoplasm (Ebashi & Endo, 1968; Sparrow *et al.*, 1970; Weber & Murray, 1973; Sobieszek & Bremel, 1975).

It has been shown that in vascular smooth muscle Ca^{2+} acts by modulating both the myosin and the actin-containing thin filaments (Bloomquist & Yaney, 1979; Marston *et al.*, 1980; Litten *et al.*, 1979). Ca^{2+} can regulate the myosin in two ways: by regulating a kinase that activates myosin by phosphorylating its regulatory light chain, and by directly binding to the myosin regulatory light chain. The presence of the former mechanism has been well documented (Sobieszek & Small, 1977; Adelstein *et al.*, 1977; Sherry *et al.*, 1978; Katzinski & Mrwa, 1980; Di Salvo *et al.*, 1979), but the direct interaction mechanism is more controversial (Chacko *et al.*, 1977; Mrwa *et al.*, 1979).

Two alternative mechanisms have been proposed for Ca^{2+} regulation of the thin filaments. Our experimentation showed that the thin filaments of vascular smooth muscle are controlled by the direct binding of Ca^{2+} to regulatory proteins that resemble troponin–tropomyosin (Marston *et al.*, 1980). Ebashi and co-workers have described a somewhat different thin-filament-linked Ca^{2+} regulatory system in gizzard and arterial smooth muscle (Hirata *et al.*, 1980). They have found that the thin filaments were unable to interact with myosin unless a small quantity of a Ca^{2+}-regulated activating factor, called leiotonin, was present. On the basis of the data

Abbreviation used: SDS, sodium dodecyl sulphate.

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presented so far, these mechanisms seem incompatible. However, it was hoped that further detailed experimentation might reconcile the apparent differences.

While working on pig aorta thin filaments we discovered that a novel 21000-mol.wt. protein component became phosphorylated when incubated with MgATP. It seemed possible that phosphorylation might have a regulatory role similar to that proposed for troponin I phosphorylation in cardiac muscle (Ray & England, 1976), and so we investigated the effects of phosphorylation of aorta thin filaments on their regulatory properties. The present paper describes our experiments, which show that phosphorylation does indeed change the regulatory properties of aorta thin filaments, but the effects are quite unlike those reported in cardiac or skeletal muscle (Ray & England, 1976; Solaro *et al.*, 1976; Buss & Stull, 1977; Jahnke & Heilmeyer, 1980). On the basis of these results we propose a hypothesis by which aorta thin-filament phosphorylation plays a role in regulating contractility in blood vessels.

Methods and materials

Preparation of pig aorta thin filaments

The basic method has been described by Marston *et al.* (1980). For the experiments described in the present paper the extraction solution was modified to 60mM-KCl, 10mM-EDTA, 5mM-$MgCl_2$, 4mM-EGTA, 20mM-4-morpholinepropanesulphonic acid (Mops)/KOH buffer, pH 7.0, 0.5mM-dithiothreitol, 10mM-ATP, 0.1mM-phenylmethanesulphonyl fluoride and 100mg of soya-bean trypsin inhibitor/l. The inclusion of proteinase inhibitors minimized degradation of the thin-filament phosphoprotein (Hathaway & Adelstein, 1979) without altering the Ca^{2+}-sensitivity of the actomyosin ATPase. Average yield was 0.41 ± 0.10mg (95% confidence limits) of thin filaments/g of tissue.

Preparation of other proteins

Rabbit skeletal-muscle myosin was prepared by the method of Perry (1955). Pig aorta myosin was prepared by the method of Sobieszek & Bremel (1975).

Measurement of phosphorylation

Aorta thin filaments at 2mg/ml were incubated at 25°C with 0–1mM-Mg[γ-^{32}P]ATP (1Ci/mol) in ATPase buffer [60mM-KCl/10mM-imidazole/HCl (pH 7.0)/5mM-$MgCl_2$/10mM-NaN_3/0.5mM-dithiothreitol] plus 0.1mM-$CaCl_2$. KF (5mM) was sometimes included to inhibit dephosphorylation (Hollander, 1971). The reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid/2% (w/v) sodium pyrophosphate. The samples were heated at 95°C for 10min and cooled on ice. Each sample was applied to a Whatman GF/C filter and washed three times with 5ml of ice-cold 5% trichloroacetic acid/1% sodium pyrophosphate and four times with 5ml of ice-cold water to remove unbound radioactivity (Daniel & Adelstein, 1976; Aksoy *et al.*, 1976). Radioactivity retained on the filter was counted by the Čerenkov method.

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 1.5mg of myosin/ml + 3mg of thin filaments/ml, in 0.5M-KCl/10mM-imidazole/HCl (pH 7.0)/5mM-$MgCl_2$/10mM-NaN_3/0.2mM-dithiothreitol at 4°C. The mixture was left for 6h so that the two proteins could hybridize and was then dialysed overnight against ATPase buffer at 4°C. Myosin and thin-filament controls were treated in the same way.

Assay of ATPase activity

The ATPase activity of reconstituted actomyosin was measured at 25°C in ATPase buffer plus 2mM-EGTA and 0–2mM-$CaCl_2$ (see below under 'Maintenance of Ca^{2+} concentration'). The reaction was started by adding MgATP to 2mM and terminated after 0, 1, 2 and 3min 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 5min, and so the rate was calculated by a least-squares fit of the P_i-versus-time data.

To compare the ATPase activities of reconstituted actomyosins containing phosphorylated and unphosphorylated thin filaments a batch of actomyosin (1.5mg of myosin/ml, 3.0mg of thin filaments/ml) was divided into two portions. The first was phosphorylated by incubation at 25°C with 12mM-MgATP and 50μM-$CaCl_2$ in ATPase buffer for 20min. Phosphorylation of the thin-filament protein reached at least 85% of its maximum value during this treatment, but no phosphorylation of the skeletal-muscle myosin could be detected. The phosphorylation reaction was terminated, and ATP, ADP and P_i were removed by the addition of 0.1 vol. of washed Dowex 1 (X8). After 2min the Dowex was removed by filtration through fine nylon gauze (50μm mesh size). Two Dowex treatments were required to remove the ATP, ADP and P_i. The second portion of actomyosin was treated in exactly the same way except that MgATP was omitted, so that the thin filaments were not phosphorylated. pH was monitored throughout these treatments and was constant within 0.02 pH unit. The ATPase activity of the reconstituted actomyosin was measured imme-

diately after Dowex treatment over a range of Ca^{2+} concentrations.

Maintenance of Ca^{2+} concentration

Ca^{2+} concentrations were maintained in the range 0.01–100μM by use of Ca^{2+}/EGTA buffers (Portzehl *et al.*, 1964; Ogawa, 1968). For ATPase measurement EGTA was a constant 2mM and $CaCl_2$ concentration was varied. For Ca^{2+}-binding measurements $CaCl_2$ was a constant 102μM, this value being standardized by atomic-absorption spectroscopy, and EGTA concentration was varied. The pH was adjusted to 7.00.

The Ca^{2+} concentration was calculated by using the ion-binding computer program of Perrin & Sayce (1967) with the stability constants listed by White & Thorson (1972). The apparent Ca^{2+}–EGTA binding constant under our conditions was $3.0 \times 10^6 M^{-1}$.

Ca^{2+} binding to thin filaments

Ca^{2+} binding was measured by the double-labelling technique of Kendrick-Jones *et al.* (1970). Thin filaments (1mg) were incubated with 102μM-$^{45}CaCl_2$ and 5mM-[^{3}H]glucose as an inert volume marker. The filaments together with their bound Ca^{2+} were separated by centrifugation for 1½h at 180000*g*; 70–80% of the protein sedimented under these conditions. Pellets were resuspended in 10mM-Tris, pH8.5. The ^{3}H and ^{45}Ca radioactivities in the pellets and a sample of the supernatant were measured, from which values the amount of bound Ca^{2+} was calculated.

Gel electrophoresis

Polyacrylamide [4–30% (w/v) gradient]/0.25% SDS / 40mM-Tris / 20mM-sodium acetate / 2mM-EDTA (pH7.4) gels were run for 2h at 150V in a Pharmacia GE 2/4 electrophoresis apparatus. Polyacrylamide (8%, w/v)/6M-urea/20mM-Tris/20mM-glycine (pH8.3) slab gels were run at 350V for 1¼h.

Gels were fixed with 10% (w/v) trichloroacetic acid / 3% (w/v) sulphosalicylic acid / 30% (v/v) methanol for 40min and stained in Coomassie Blue. The position of radioactive protein bands was determined by radioautography. The stained gel was placed in contact with Agfa Osray X-ray film for 1–5 days in the dark and the film was developed in Kodak DX-80 developer. For quantitative measurement of radioactivity the protein bands were cut out, ground up, dissolved in 30% (v/v) H_2O_2 and assayed by liquid-scintillation counting (Young & Fulhorst, 1975).

Stained gels and radioautograms were scanned by using a Gilford 240 spectrophotometer with densitometer attachment.

Phosphorylated thin filaments were prepared for gel electrophoresis by incubating thin filaments (5mg/ml) with 0.5mM-[γ-^{32}P]ATP at high specific radioactivity for 3min at 25°C. Three procedures were used to stop the reaction: (1) 1% SDS was added and the sample boiled for 5min; (2) an equal volume of 5% trichloroacetic acid was added, and the protein sedimented and redissolved in SDS at 3g of SDS/g of protein; (3) solid urea was added to a final concentration of 7M.

For SDS/polyacrylamide-gel electrophoresis samples were made up to 40mM-Tris/20mM-sodium acetate/2mM-EDTA/12% (v/v) glycerol/2% (v/v) 2-mercaptoethanol. All three methods produced identical patterns of protein bands on electrophoresis, but there was often substantial loss of protein-bound radioactivity when method (1) was used. For 6M-urea/pH8.3 gel electrophoresis samples prepared by method (3) were used without further treatment.

Results

Pig aorta thin filaments can be phosphorylated

When Ca^{2+}-regulated aorta thin filaments were incubated with 1mM-Mg[γ-^{32}P]ATP, ^{32}P was incorporated into the thin filaments. It was retained through repeated washings in 5% trichloroacetic acid and was therefore probably covalently bound to thin-filament proteins. No radioactivity was incorporated when thin filaments were incubated in Mg[α-^{32}P]ATP, and thus only the P_i (or PP_i) moiety of ATP was incorporated.

At 25°C at pH7.0, maximum incorporation was obtained in about 40min (Fig. 1) and was followed

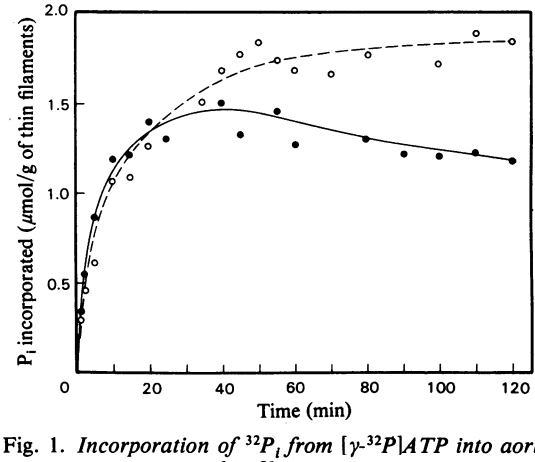

Fig. 1. *Incorporation of $^{32}P_i$ from [γ-^{32}P]ATP into aorta thin filaments*

Incubation conditions were as follows: ATPase buffer, 1mM-Mg[γ-^{32}P]ATP (sp. radioactivity about 1Ci/mol), 0.1mM-$CaCl_2$, pH7.0, 25°C. For full details see the text. ●, No further additions; ○, plus 5mM-KF.

by a slow decline, presumably due to phosphatase activity. If 5 mM-KF, a phosphatase inhibitor (Hollander, 1971), was included the maximum incorporation was 15–20% greater and was maintained for at least 3 h. Maximum incorporation determined with seven preparations in the presence of 5 mM-KF was 1.8 ± 0.1 μmol of P_i/g of filaments (95% confidence limits given). When phosphorylated thin-filament proteins were separated by electrophoresis in 0.25% SDS/polyacrylamide gels the radioactivity was mainly located in a band with a molecular weight of 21000 (Fig. 3).

The phosphorylation of aorta thin filaments was catalysed by a factor present in the thin-filament preparation, either as a component of the thin filament or as an impurity of the preparation. We sedimented the thin filaments by high-speed centrifugation (180000 *g* for 1½h). The initial rate of $^{32}P_i$

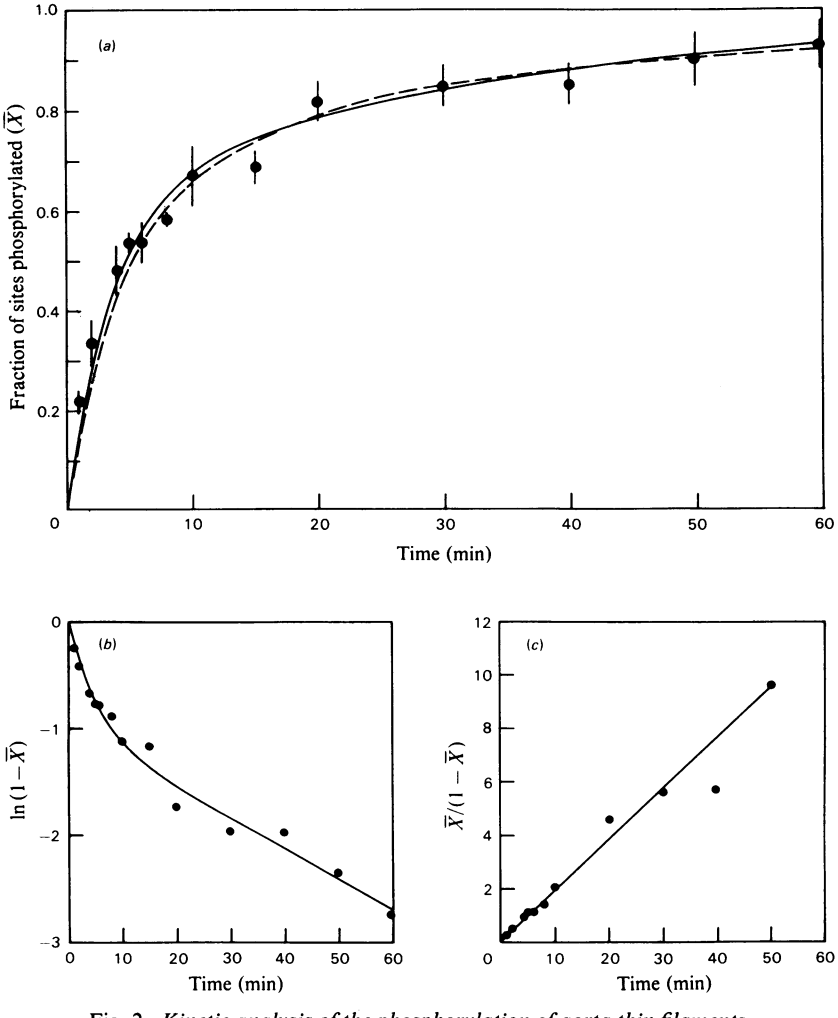

Fig. 2. *Kinetic analysis of the phosphorylation of aorta thin filaments*

Phosphorylation was measured in the presence of 5 mM-KF, the conditions being as for Fig. 1. For full details see the text. Results from seven experiments were normalized by assuming 100% incorporation after 3 h incubation. The means ($\bar{X}$) and 95% confidence limits are plotted. (*a*) Direct plot of $\bar{X}$ versus time. ——, Calculated fit to the data on the basis of two classes of sites; — —, calculated fit assuming a second-order process. (*b*) Plot of $\ln(1-\bar{X})$ versus time, showing the non-linearity. ——, Calculated best fit assuming two classes of sites:

$$(1-\bar{X}) = 0.4^{e^{-0.028t}} + 0.6^{e^{-0.28t}}$$

(*c*) Plot of $\bar{X}/(1-\bar{X})$ versus time. Estimated k_2 is 0.19 min^{-1}, equivalent to a second-order rate constant of 0.105 $min^{-1} \cdot$ (μmol of P_i/g of thin-filament protein)$^{-1}$ on the basis of 1.8 μmol/g of phosphorylation sites.

incorporation by the resuspended pellet fraction was 1.0 ± 0.4 times the rate from whole thin filaments, and the maximum $^{32}P_i$ incorporation was 0.9 ± 0.3 times that of whole thin filaments (95% confidence limits given, ten measurements). Since the pelleted thin filaments occupied only one-hundredth of the original solution volume, we concluded that both the kinase activity and the 21000-mol.wt. substrate protein were bound to the aorta thin filaments.

Kinetics of phosphorylation

The intrinsic thin-filament kinase activity had a maximum initial rate of 0.4 ± 0.1 nmol of P_i incorporated/min per mg of thin filaments (95% confidence limits, seven protein preparations) with 1 mM-MgATP. The initial rate depended on MgATP concentration in a hyperbolic manner, from which we estimated a K_m for MgATP of $32 \pm 14\,\mu$M (mean and 95% confidence limits, three preparations).

With a saturating MgATP concentration, half-maximal $^{32}P_i$ incorporation into the aorta thin filaments was obtained in $4\frac{1}{2}$min at 25°C, but the time course was not a simple exponential, even in the presence of 5 mM-KF (Fig. 2). Semi-logarithmic plots of the data (ln[unoccupied phosphorylation sites] versus time) were curved, with the slope decreasing with time (Fig. 2*b*).

Two models can account for our observations. The first model assumes heterogeneous phosphorylation sites. A good fit to the data could be obtained by two exponential time courses:

$$(1-\bar{X}) = A\,e^{-k_1 t} + B\,e^{-k_2 t}$$

where $\bar{X}$ is the fraction of phosphorylation sites occupied at time t, k_1 and k_2 are first-order rate constants and A and B represent the relative amplitudes of the two exponentials. The best fit was estimated with A and B about equal, $k_1 = 0.28\,\text{min}^{-1}$ and $k_2 = 0.028\,\text{min}^{-1}$ (see Fig. 2*b*). Alternatively, we assumed a second-order reaction: rate of P_i incorporation = $k_2 \cdot$[unoccupied phosphorylation sites]^2. The time course is given by $\bar{X}/(1-\bar{X}) = k_2 t$, and data plotted according to this equation (Fig. 2*c*) gave a linear plot with $k_2 = 0.105\,\text{min}^{-1} \cdot (\mu\text{mol of } P_i/\text{g of thin filaments})^{-1}$.

A second-order rate equation could be the consequence of both kinase and its substrate being tightly bound to the thin filament.

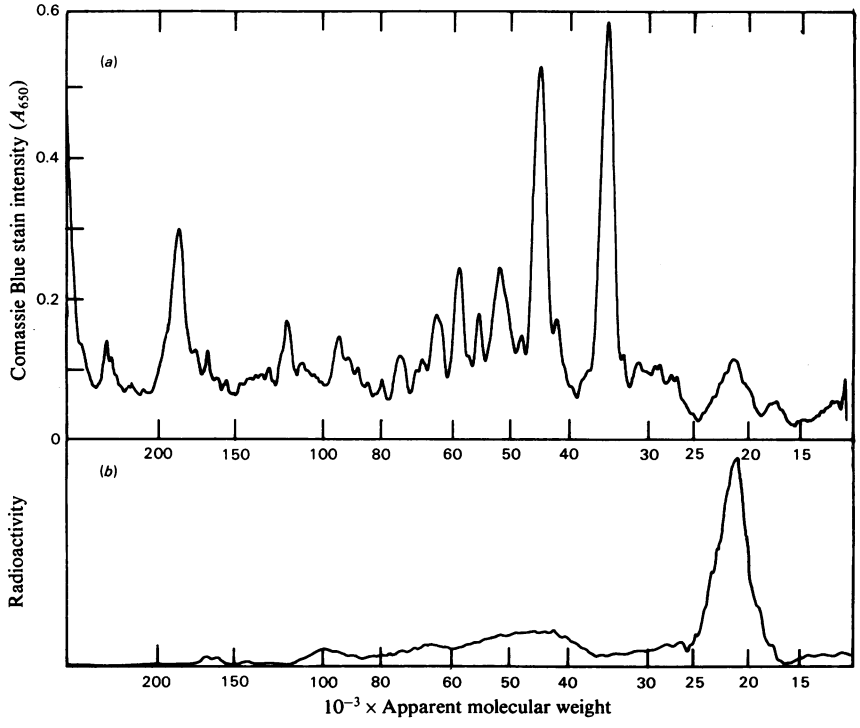

Fig. 3. *Densitometer scans of electrophoretograms of phosphorylated aorta thin filaments in polyacrylamide (4–30% gradient)/0.25% SDS gel*

The electrophoresis was performed with 40 μg of aorta thin filaments. For full details see the text. (*a*) Gel stained in Coomassie Blue and scanned at 650nm. The molecular-weight scale was calibrated by using standard proteins. (*b*) Scan of a radioautogram made from the gel shown in (*a*).

Both models accurately account for the observed time course (Fig. 2); to find out which mechanism operates in aorta thin-filament phosphorylation will require evidence from independent experiments. Preliminary experiments indicated that the initial rate and plateau of phosphate incorporation were decreased by lowering $[Ca^{2+}]$ to 0.01 μM and increased by adding 1 μM-cyclic AMP.

Dephosphorylation of aorta thin filaments

Loss of $^{32}P_i$ from the phosphorylated thin-filament protein could be measured under conditions where $^{32}P_i$ incorporation was prevented. We stopped $^{32}P_i$ incorporation by three methods: removal of Mg[γ-^{32}P]ATP by rapid gel filtration, inhibition of kinase by adding EDTA to 10 mM, and displacement of Mg[γ-^{32}P]ATP by a 12-fold excess of unlabelled MgATP. We found that the thin filaments were slowly dephosphorylated in the absence of any added enzymes; half the radioactivity was lost in 3 h at 25°C, and over 90% was removed after the preparation had been left overnight. Since the reaction was so slow, there were insufficient data for a full kinetic analysis, but on average the data fitted a single-exponential decay better than a second-order decay. The apparent dephosphorylation rates were to some extent influenced by the different methods. The highest rate was obtained with the gel-filtration method, an intermediate rate was obtained with inhibition by EDTA and the 'chase' with excess of unlabelled ATP gave the lowest rate of dephosphorylation (0.0048, 0.0036 and 0.0022 min^{-1} respectively, assuming a single-exponential decay).

Phosphorylated protein

Freshly phosphorylated aorta thin filaments were analysed by electrophoresis in SDS (0.25%)/polyacrylamide (4–30% gradient) gels. The major protein components were of mol.wt. 42000 (actin) and of mol.wt. 37000 (this band probably includes tropomyosin plus other proteins of the same molecular weight). In the low-molecular-weight range there were peptides of 26000, 21000 and 17000 mol.wt. (Fig. 3) (Marston *et al.*, 1980). Radioautography of the gels showed that the $^{32}P_i$ was mostly confined to a single band, corresponding to the 21000-mol.wt. protein (Fig. 3). At least 75% of radioactivity was in this band after 3 min incubation at 25°C. Prolonged incubation in [γ-^{32}P]ATP resulted in numerous minor bands incorporating P_i. Variable amounts of a 9000-mol.wt. band of radioactivity were sometimes seen, which may have been a degradation product of the 21000-mol.wt. protein. The phosphorylated thin-filament protein is distinguishable from the 20000-mol.wt. phosphorylated light chain of aorta myosin on gradient gels, but on homogeneous 7½% polyacrylamide gels the bands were not sharp enough to enable differentiation between the two.

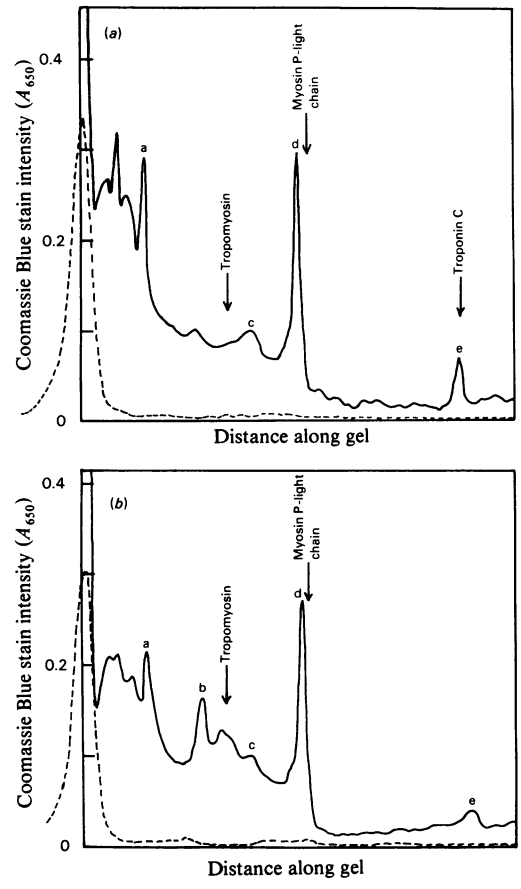

Fig. 4. *Densitometer scans of electrophoretograms of phosphorylated aorta thin filaments in polyacrylamide (8%)/6 M-urea (pH 8.3) gel*

The electrophoresis was performed with 300 μg of aorta thin filaments in the presence of 10 mM-EGTA (*a*) or 10 mM-$CaCl_2$ (*b*). For full details see the text. ——, Scan of protein stained with Coomassie Blue; – – – –, scan of radioactivity located by radioautography. Letters refer to the bands previously described in aorta thin filaments (Marston *et al.*, 1980). Arrows give the location of pure aorta tropomyosin, aorta myosin 20000-mol.wt. regulatory light chain and skeletal-muscle troponin C in this system.

When the phosphorylated thin filaments were separated by electrophoresis in polyacrylamide (8%)/6 M-urea/20 mM-Tris/glycine (pH 8.3) gels in the presence of 10 mM-$CaCl_2$ or 10 mM-EGTA, the phosphorylated protein did not migrate into the gel (Fig. 4). A very small fraction (0.6%) of the radioactivity migrated with the mobility of myosin P-light chain, presumably due to myosin and myosin

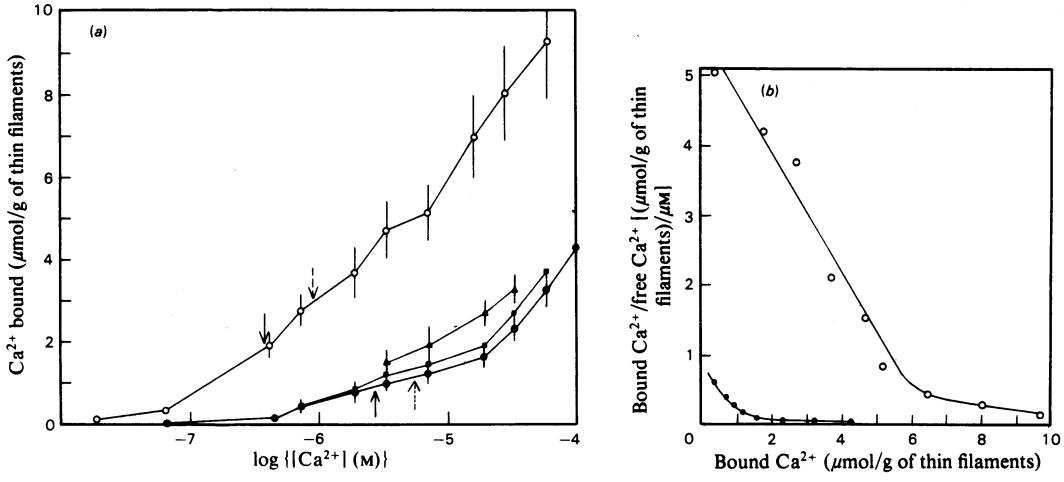

Fig. 5. *Ca^{2+} binding by phosphorylated and unphosphorylated aorta thin filaments*

For full details see the text. (*a*) Dependence of Ca^{2+} binding on log[Ca^{2+}]. ○, Phosphorylated thin filaments; ●, unphosphorylated thin filaments; ▲, phosphorylated thin filaments + 5 mM-KF; ■, unphosphorylated thin filaments + 5 mM-KF. ATPase buffer (pH 7.0) at 25°C was present. Points are means and 95% confidence limits for seven experiments with different protein preparations. ↓ indicates [Ca^{2+}] where skeletal-muscle myosin–aorta thin filament ATPase activity is half-maximally activated; ↓ indicates where they are fully activated (see Fig. 6). (*b*) Data from (*a*) replotted as a Scatchard plot.

kinase impurities in our preparation. No other radioactive protein bands were observed.

Under normal conditions the phosphorylated protein remained bound to the thin filaments. The thin-filament proteins were dissociated under denaturing conditions, and a partial purification of the phosphorylated protein could be achieved by gel filtration on Sephacryl S-300 in the presence of 6 M-urea at pH 6.8.

Phosphorylation increases aorta thin-filament Ca^{2+} binding

In our previous study (Marston *et al.*, 1980) we found that preincubation of aorta thin filaments with 1 mM-MgATP increased the amount of Ca^{2+} bound at high affinity ($K \simeq 10^6 M^{-1}$). Since we now know that this treatment causes phosphorylation of the thin filaments, it seemed likely that the increase in Ca^{2+} binding was a consequence of phosphorylation.

We therefore examined aorta thin-filament Ca^{2+} binding more closely. Phosphorylation was decreased to a minimum basal value by prolonged dialysis against ATPase buffer. The unphosphorylated thin filaments bound only about 0.8–1.5 μmol of Ca^{2+}/g at high affinity (binding constant 0.5×10^6–$1.5 \times 10^6 M^{-1}$) plus up to 6 μmol of Ca^{2+}/g bound at a much lower affinity (binding constant about $10^4 M^{-1}$) (Fig. 5). The presence of the low-affinity component made it impossible to determine the quantity and affinity of the high-affinity sites more accurately.

When thin filaments were incubated with 1 mM-MgATP for 30 min before measurement of Ca^{2+} binding the quantity of Ca^{2+} bound was at least 4-fold greater (Fig. 5). At Ca^{2+} concentrations up to 16 μM the Scatchard plot of the data was linear (Fig. 5*b*). The data in the range 0.06–16 μM were therefore analysed by a least-squares fit to the equation for a single class of binding sites (Wilkinson, 1961). Maximum binding was 6.3 ± 0.3 (S.E.M.) μmol/g and the binding constant was $0.61 \times 10^6 \pm 0.07 \times 10^6 M^{-1}$. There was also evidence for further Ca^{2+} binding at a lower affinity, which may correspond to non-specific binding to the actin (Hirata *et al.*, 1980). The ATP-dependent increase in Ca^{2+} binding correlated with phosphorylation of the thin filaments under all conditions tested (Table 1). Ca^{2+} binding was not increased by preincubation with Mg[βγ-imido]ATP, an ATP analogue that did not phosphorylate the thin filament, nor was it increased when ATP was added immediately before measurement of the Ca^{2+} binding, with omission of the preincubation step. On the other hand, if the thin filaments were preincubated in 1 mM-ATP, which would result in phosphorylation, but Ca^{2+} binding was measured in the absence of ATP, Ca^{2+} binding was high. If the phosphorylated thin filaments were dephosphorylated by prolonged dialysis in the absence of ATP the Ca^{2+} binding was, once again, low.

Table 1. *Ca^{2+} binding by aorta thin filaments under diverse conditions*

Conditions were as follows: 50mM-KCl, 10mM-imidazole/HCl buffer, pH 7.0, 5mM-$MgCl_2$, 10mM-NaN_3, 102μM-$^{45}CaCl_2$, 0.11mM-EGTA, 5mM-[^{3}H]glucose, 25°C. All measurements were made at a constant 6.3μM-Ca^{2+} (pCa 5.2). Means ± 95% confidence limits of five to ten measurements are given. The degree of phosphorylation was measured in parallel experiments with [γ-^{32}P]ATP under conditions identical with those for the $^{45}Ca^{2+}$-binding measurements.

Treatment	Ca^{2+} bound at 6.3μM (μmol/g)	Degree of phosphorylation (% of maximum)
No treatment	1.4 ± 0.2	0
Preincubation, and measurement in the presence of 1mM-MgATP	5.0 ± 0.5	100
Preincubation, and measurement in the presence of 1mM-Mg[$\beta\gamma$-imido]ATP	1.41 ± 0.5	0
1mM-MgATP, no preincubation	2.5 ± 0.5	10–40
1mM-MgATP preincubation, then ATP removed during measurement*	3.5 ± 0.3	80–90
Phosphorylated, then dephosphorylated	2.7 ± 1.2	5–15

* ATP was removed by passing the thin filaments through two Sephadex G-25 columns (1.5cm × 10cm); the process took 9min overall.

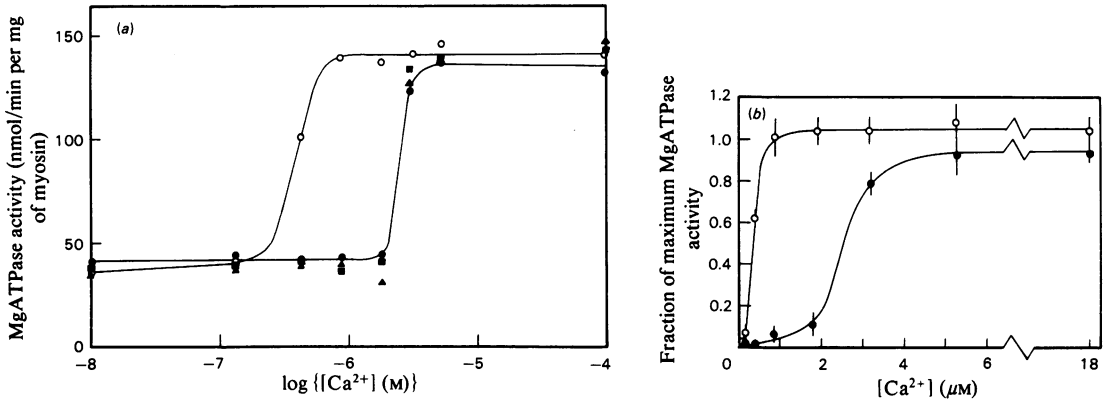

Fig. 6. *Dependence of the activation of myosin ATPase activity by aorta thin filaments on Ca^{2+} concentration*

Activation is indicated by total ATPase activity minus myosin ATPase activity. ATPase buffer, (pH 7.0, at 25°C), 2mM-MgATP, 1.5mg of skeletal-muscle myosin/ml and 3mg of thin filaments/ml were present. ○, Phosphorylated; ●, unphosphorylated; ▲, phosphorylated + 5mM-KF; ■, unphosphorylated + 5mM-KF. For full details see the text. (*a*) Dependence of activation of ATPase activity on log[Ca^{2+}]; results are for one experiment. (*b*) Dependence of activation of ATPase activity on Ca^{2+} concentration. The data from several experiments were normalized. Thus 0% = ATPase activity at 0.01μM-Ca^{2+}, 100% = ATPase activity at 100μM-Ca^{2+}. Points are means ± S.D. for four experiments with different thin filament preparations.

Although we could not obtain high Ca^{2+} binding without phosphorylation, we did find conditions where the thin filaments were phosphorylated but the quantity of Ca^{2+} bound was still low. This was observed when 5mM-KF was present during preincubation in MgATP and binding measurement (Fig. 5). KF had no effect on the Ca^{2+} binding of unphosphorylated thin filaments (Fig. 5), nor did it inhibit thin-filament phosphorylation (Fig. 1). Thus fluoride appears to uncouple the increase in Ca^{2+} binding from phosphorylation.

Effect of aorta thin-filament phosphorylation on actomyosin ATPase activity

From our previous work (Marston *et al.*, 1980) we know that unphosphorylated aorta thin filaments can activate skeletal-muscle myosin Mg-ATPase. The activation is Ca^{2+}-regulated and is half-maximal at 2.7μM-Ca^{2+} (Fig. 6). Since phosphorylation of aorta thin filaments has a large effect on Ca^{2+} binding (Fig. 5), and since, presumably, the Ca^{2+} regulation is a consequence of Ca^{2+} binding to

the thin filaments, it is likely that phosphorylation of the thin filaments would significantly alter their Ca^{2+}-dependent activation of myosin MgATPase.

To test this hypothesis synthetic actomyosins containing 1.5 mg of skeletal-muscle myosin/ml and 3.0 mg of phosphorylated or unphosphorylated thin filaments/ml were prepared as described in the Methods and materials section and ATPase activity was measured over a range of Ca^{2+} concentrations (Fig. 6). When the thin filaments had been phosphorylated the ATPase activity was activated by much lower Ca^{2+} concentrations: half-maximal activity was obtained at 0.35 μM-Ca^{2+}, compared with 2.7 μM for the unphosphorylated thin filaments. This was the only significant effect of phosphorylation on ATPase activity; at high Ca^{2+} concentrations (> 10 μM) the ATPase activities were almost the same and the curves exhibited positive cooperativity whether the thin filaments were phosphorylated or not (Fig. 6*b*). The effect could not be observed in the presence of 5 mM-KF (Fig. 6*a*).

It is conceivable that this shift in Ca^{2+} concentration required for activation was an artifact introduced by the treatment given to the actomyosin: preincubation with ATP and removal of ATP with Dowex 1 (X8) ion-exchanger. We minimized the possibility of introducing artifacts by giving as nearly as possible identical treatments to the phosphorylated and unphosphorylated actomyosins and by ensuring that the pH did not vary by more than 0.02 pH unit and that the myosin was not phosphorylated.

The effect of thin-filament phosphorylation on the Ca^{2+}-activated skeletal-muscle myosin–aorta thin-filament MgATPase activity can be demonstrated by a completely different experiment that does not involve any preincubation or treatment with ion-exchanger. It will be noted from Fig. 6 that at Ca^{2+} concentrations in the range 1–2 μM the MgATPase activity is highly dependent on whether or not the thin filaments are phosphorylated. When the time course of MgATPase of an untreated skeletal-muscle myosin–aorta thin filament hybrid was measured at a Ca^{2+} concentration of 1.8 μM it was observed that the rate was initially that expected for unphosphorylated thin filaments (Fig. 7). After 6 min the rate began to increase, reaching the rate for phosphorylated thin filaments after 11 min. In contrast, the MgATPase rates measured at 0.01 μM- or 100 μM-Ca^{2+} remained roughly constant for the whole 20 min of the experiment. This result is precisely what was predicted if the Ca^{2+} concentration required for activation was lowered by phosphorylation as found in the direct experiments (Fig. 6), and the time scale of the change in MgATPase activity is compatible with the observed rate of P_i incorporation into aorta thin filaments (Fig. 2: half-maximal incorporation in 4½ min).

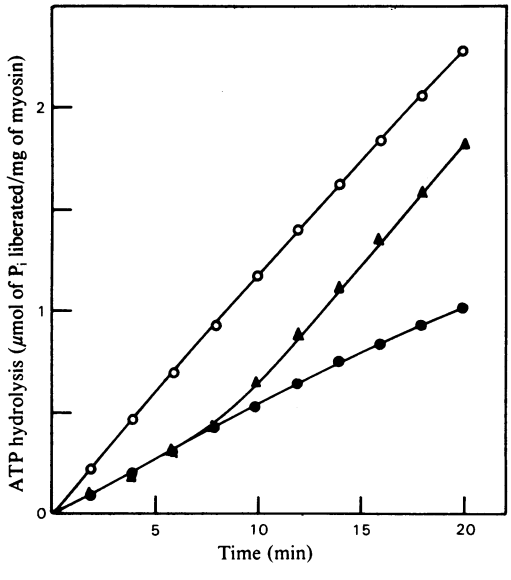

Fig. 7. *Time course of ATP hydrolysis by a skeletal-muscle myosin/aorta thin filament mixture*
Hydrolysis of ATP by 1.5 mg of skeletal-muscle myosin/ml + 3 mg of aorta thin filaments/ml with 5 mM-MgATP and ATPase buffer (pH 7.0, at 25°C). ●, 0.01 μM-Ca^{2+}; ▲, 1.8 μM-Ca^{2+}; ○, 100 μM-Ca^{2+}. For full details see the text.

Discussion

Mechanism of phosphorylation of aorta thin filaments

We have found that preparations of Ca^{2+}-regulated thin filaments from pig aorta smooth muscle are capable of covalently incorporating P_i from MgATP (Fig. 1). The P_i was mainly incorporated into a single protein, of mol.wt. 21 000 (Fig. 3). This phosphoprotein appears to be an integral part of the thin filament, since it co-sedimented with the thin filaments during high-speed centrifugation and could not be separated chromatographically, except under denaturing conditions (pH 6.8, 0.2 M-KCl, 0.1 mM-EGTA, 6 M-urea). Thin filaments incorporated a maximum of 1.8 μmol of P_i/g, with half-maximal incorporation taking 4½ min at 25°C (Fig. 1).

From densitometry of SDS/polyacrylamide-gel electrophoretograms we have estimated that there was 1 mol of $^{32}P_i$ incorporated for every 2–4 mol of actin. We do not know how many P_i molecules were incorporated into each molecule of 21 000-mol.wt. protein. Kinetic analysis of P_i incorporation suggested that there may have been two (or more) kinetically distinguishable phosphorylation sites (Fig. 2), but we have been unable to obtain any direct evidence to support this.

In skeletal-muscle and cardiac-muscle thin filaments tropomyosin, troponin I and troponin T are phosphorylated *in vivo* or can be phosphorylated *in vitro* by protein kinases (Perry, 1979; Stull *et al.*, 1980). We have previously identified tropomyosin and proteins that behave like troponin T and troponin C in aorta thin filaments (Fig. 4) (Marston *et al.*, 1980). However, the phosphoprotein can be distinguished from these on the basis of molecular weight (Fig. 3) or electrophoretic mobility in 6M-urea (pH 8.3)/polyacrylamide gel (Fig. 4). The aorta thin-filament phosphoprotein is thus not analogous to the phosphoproteins observed in skeletal-muscle thin filaments.

The aorta thin-filament phosphoprotein was distinguishable from the 20000-mol.wt P-light chain of aorta myosin (a minor contamination in thin-filament preparations) on electrophoresis in polyacrylamide (4–30% gradient)/0.25% SDS gel and in polyacrylamide (8%)/6M-urea (pH 8.3) gel (Figs. 3 and 4). It should be noted that the two phosphoproteins were not always distinguishable on SDS/polyacrylamide-gel electrophoresis in homogeneous gels. In a number of experiments with skinned fibres on whole muscle, the 20000-mol.wt. phosphorylated protein band on SDS/polyacrylamide-gel electrophoresis has been assigned wholly to myosin P-light chain. Our finding suggests that this assumption can lead to erroneous interpretation if the thin-filament phosphoprotein was also phosphorylated (Guatieri & Janis, 1977; Barron *et al.*, 1979; Cassidy *et al.*, 1979).

Phosphoprotein kinase activity was present in the preparation of aorta thin filaments (Fig. 1). It is likely that the kinase was bound as an integral component of the aorta thin filaments, since when thin filaments were sedimented the kinase activity was retained in the pellet of thin filaments. There is evidence for an endogenous protein kinase bound to the regulatory proteins in cardiac muscle (Reddy, 1976).

We do not know very much about the dephosphorylating activity in our thin-filament preparation. Dephosphorylation of aorta thin filaments was slow ($0.005\,min^{-1}$) and, apparently, not greatly inhibited by 10mM-ATP (Hsiao *et al.*, 1978). The possibility of dephosphorylation by proteolysis of the 21000-mol.wt. protein has not been excluded. It is possible that most of the phosphatase activity has been removed during the preparation procedure.

Modification by phosphorylation of the Ca^{2+}-sensitive regulatory properties of the thin filaments

The phosphorylation of the 21000-mol.wt. protein of aorta thin filaments became of greater interest when it was discovered that phosphorylation has profound effects on the regulatory properties of the thin filaments. Earlier work (Marston *et al.*, 1980) had shown that Ca^{2+} binding to aorta thin filaments was influenced by the presence of MgATP. A re-examination of the data has shown us that an increase in Ca^{2+} binding by the thin filaments was coupled to phosphorylation, since, with a variety of treatments, we could only obtain high Ca^{2+} binding when the thin filaments were phosphorylated (Table 1).

The effect of phosphorylation on Ca^{2+} binding was an increase in the quantity of high-affinity Ca^{2+}-binding sites (i.e. $K \simeq 10^6 M^{-1}$) from 0.8–1.5 μmol/g to 6.3 μmol/g (Fig. 5).

This effect is quite different from the effect of phosphorylation of skeletal-muscle troponin T or cardiac-muscle troponin I, where Ca^{2+} binding is unchanged or the affinity is decreased (Buss & Stull, 1977; Jahnke & Heilmeyer, 1980).

For every molecule of P_i incorporated about three new high-affinity Ca^{2+} binding sites appeared in the aorta thin filament (cf. Figs. 1 and 5). This stoicheiometry suggests that P_i was acting as an allosteric effector inducing conformational changes rather than directly (i.e. Ca^{2+} binding to the P_i moiety). This hypothesis was supported by our observation (Fig. 5) that phosphorylation and increase in Ca^{2+} binding could be uncoupled from each other in the presence of 5mM-KF. We do not yet know which protein carried the new high-affinity Ca^{2+}-binding sites. In striated (i.e. skeletal and cardiac)-muscle thin filaments troponin C is the only Ca^{2+}-binding protein, and phosphorylation of the other troponin subunits can alter the Ca^{2+}-binding properties of troponin C (Jahnke & Heilmeyer, 1980). Our thin-filament preparations contain a troponin C-like protein (Fig. 4). The quantity of troponin C-like protein is at least 1.5 μmol/g (Marston *et al.*, 1980), sufficient to account for all the high-affinity Ca^{2+}-binding sites in phosphorylated thin filaments if there are four binding sites per molecule (Potter & Gergely, 1974), so it is possible that phosphorylation of the 21000-mol.wt. protein induces a conformational change in the troponin C-like protein that changes the affinity of some of its Ca^{2+}-binding sites from low to high (Bremel & Weber, 1972).

Aorta thin filaments activated skeletal-muscle myosin MgATPase activity, and the degree of activation was regulated by Ca^{2+} (Marston *et al.*, 1980; Fig. 6). Phosphorylation of the thin filaments was associated with a substantial decrease in the Ca^{2+} concentration required to activate the actomyosin ATPase activity (Fig. 6). For phosphorylated thin filaments half-maximal activation of the ATPase activity required (0.35 μM-Ca^{2+}, compared with 2.7 μM-Ca^{2+} for unphosphorylated thin filaments. The time course of this increase in sensitivity to activation by micromolar Ca^{2+} concentrations was consistent with the effect being a consequence of

phosphorylation of the 21000-mol.wt. protein (Fig. 7). The change in Ca^{2+}-sensitivity was the only effect of phosphorylation, since the ability of thin filaments to activate skeletal-muscle myosin MgATPase activity at saturating Ca^{2+} concentrations (10μM) was not altered (Figs. 6 and 7).

If Ca^{2+} binding to aorta thin filaments is a prerequisite for Ca^{2+} activation of the actomyosin MgATPase activity, it is reasonable to propose that the effect of phosphorylation on the Ca^{2+} concentration required for activation (Fig. 6) is due to the increased Ca^{2+} binding (Fig. 5). We cannot prove this, but the observation that 5mM-KF uncouples both the increase in Ca^{2+} binding (Fig. 5) and the increase in sensitivity to 1.8μM-Ca^{2+} (Fig. 6) from phosphorylation is compatible with the hypothesis. However, there was not a simple relationship between Ca^{2+} binding and MgATPase activity; thus unphosphorylated thin filaments were fully activated when 1.15μmol of Ca^{2+}/g bound and half-maximally activated when 0.9μmol of Ca^{2+}/g bound, whereas the phosphorylated thin filaments were fully activated when 3.0μmol of Ca^{2+}/g bound and half-maximally activated when 1.8μmol of Ca^{2+}/g bound (see arrows on Fig. 5). These observations suggest phosphorylation may produce other effects in addition to increasing Ca^{2+} binding at regulatory sites.

The regulatory effects of phosphorylation on aorta thin filaments are unlike the effects of phosphorylation on striated-muscle thin-filament regulatory proteins. Phosphorylation of cardiac-muscle troponin I (Solaro *et al.*, 1976; Ray & England, 1976) or skeletal-muscle troponin T (Jahnke & Heilmeyer, 1980) produces much smaller changes in the Ca^{2+} concentration requirement for half-maximal activation of ATPase activity (2-fold compared with 8-fold; Fig. 6), and these are in the opposite direction to the changes we found in aorta thin filaments.

We have investigated whether the thin-filament regulatory system we have discovered in vascular smooth muscle (Marston *et al.*, 1980) and its modulation by phosphorylation described in the present paper could account for any of the observations made by Ebashi and his colleagues (Ebashi *et al.*, 1975, 1977; Mikawa *et al.*, 1977*a*,*b*, 1978; Hirata *et al.*, 1977, 1980). They found that vascular smooth-muscle thin filaments contained actin-bound proteins of mol.wts. 80000 and 20000, which they called leiotonins A and C. In the absence of leiotonin the actin filaments did not activate vascular myosin, whereas in the presence of small amounts of leiotonin actin filaments were active and Ca^{2+}-regulated. We noted that, according to our calculations, the 'activating solution' used by Ebashi and his colleagues (see Table 2) would contain only 6μM-Ca^{2+} at pH 6.8. This would be only just enough to activate our thin filaments at pH 7.0 if they were not phosphorylated (Fig. 6). When we repeated our experiments under the incubation conditions described by Hirata *et al.* (1977), we found that phosphorylated thin filaments were activated in 'activating solution' but unphosphorylated thin filaments were not (Table 2). Could it be that the 'leitonin actin' described by Ebashi and his colleagues corresponds to our phosphorylated thin-filament preparation and that the activating effect of 'leiotonin' on aorta thin filaments at 6μM-Ca^{2+} at pH 6.8 is a consequence of phosphorylation induced by 'leiotonin'? Our thin-filament preparations do contain protein components in the 80000-mol.wt. and 15000–20000-mol.wt. regions that might be leiotonins A and C (Fig. 3). However, it should be noted that leiotonin C is reported to be an acidic Ca^{2+}-binding protein, of mol.wt. 18000, that functionally resembles calmodulin (Hirata *et al.*, 1980), and is thus quite different from our 21000-mol.wt. phosphoprotein (see Figs. 3 and 4).

Physiological role for thin-filament phosphorylation

The mechanism by which a 21000-mol.wt. component of the thin filament of vascular smooth muscle is phosphorylated, leading to an approxi-

Table 2. *Effect of phosphorylation on Ca^{2+} activation of aorta thin filament–skeletal-muscle myosin MgATPase activity under the conditions described by Ebashi and his colleagues (Hirata et al., 1977)*

ATPase activity was measured at 25°C with aorta thin filament–skeletal-muscle myosin hybrids prepared as described in the Methods and materials section.

		ATPase activity (nmol/min per mg)	
Buffer	[Ca^{2+}] (μM)	3mg of thin filaments/ml + 1.5mg of skeletal-muscle myosin/ml	3mg of phosphorylated thin filaments/ml + 1.5mg of skeletal-muscle myosin/ml
Ebashi's buffer, pH 6.8*			
'Relaxing'	0.01	24	33
'Activating'	6.2	30	131

* 20mM-Tris/maleate, pH 6.8, 8mM-$MgCl_2$, 50mM-KCl, 0.4mM-EGTA, 1mM-MgATP ('relaxing'); 'activating' buffer contained in addition 0.36mM-$CaCl_2$.

mately 4-fold increase in Ca^{2+} binding and an 8-fold decrease in the Ca^{2+} concentration required to switch on the thin-filament activation of myosin MgATPase, has no parallel in skeletal or cardiac muscle. Is this mechanism involved in the regulation of vascular smooth-muscle contraction?

We know that vascular smooth muscle has dual Ca^{2+} regulation (Litten *et al.*, 1979; Bloomquist & Yaney, 1979; Marston *et al.*, 1980), and consequently both the thin filaments and the myosin need to be activated for contraction to occur. Additional modulation of the thin-filament regulatory system by phosphorylation increases the complexity of regulation. With multiple regulatory systems there exists the capability to regulate contractility rather precisely; how could the regulatory systems interact?

Myosin phosphorylation is probably obligatory for activation of vascular myosin (Sherry *et al.*, 1978; Di Salvo *et al.*, 1979; Katzinski & Mrwa, 1980); in our hands the myosin kinase is half-maximally activated by 0.8 μM-Ca^{2+} (R. M. Mayers, unpublished work), so myosin should be half-maximally activated at 0.8 μM-Ca^{2+} or less (depending on the rate of dephosphorylation). The Ca^{2+} concentration required to activate the thin filaments depends on the degree of phosphorylation, and is variable between 0.36 μM- and 2.7 μM-Ca^{2+} (Fig. 6). On the basis of these data we propose a model in which all three regulatory mechanisms play a role in controlling contractility. (1) At low degrees of thin-filament phosphorylation, the thin filaments need more Ca^{2+} to switch on than does myosin kinase; myosin is thus always active when thin filaments are switched on by increasing $[Ca^{2+}]$, and so contractility depends on the thin-filament regulatory system. (2) At a high degree of thin-filament phosphorylation the myosin kinase is switched on at a higher $[Ca^{2+}]$ than are the thin filaments, so activation of contraction by increasing $[Ca^{2+}]$ depends on the myosin regulatory system. (3) At intermediate degrees of thin-filament phosphorylation, either form of regulation may predominate and the $[Ca^{2+}]$ needed for half-maximal activation can vary within the range 0.8–2.7 μM.

The concentration of Ca^{2+} in the activated muscle cell has been estimated to be in the range 0.5–5 μM (Kretsinger, 1979), so a physiological role for our proposed scheme is plausible.

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