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Phosphatase-Mediated Modulation of Actin–Myosin Interaction in Bovine Aortic Actomyosin and Skinned Porcine Carotid Artery (41981)

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Abstract. Since the Ca^{2+}-regulatory mechanism for actin–myosin interaction in smooth muscle involves phosphorylation of the 20,000-Da myosin light chains, it was hypothesized that such interaction should be influenced by myosin phosphatase. Accordingly, we studied the effects of an aortic myosin light-chain phosphatase on Ca^{1+}-dependent actin–myosin interaction in detergent-skinned porcine carotid artery and bovine aortic native actomyosin. In skinned preparations, the aortic phosphatase (16 U/ml) markedly inhibited the rate of isometric contraction in low Ca^{2+} (6.8×10^{-7} *M*) and responsiveness to Ca^{2+} (force attained with 6.8×10^{-7} Ca^{2+}/force attained with 1.6×10^{-6} *M* Ca^{2+}), whereas relaxation was accelerated. Ca^{2+}-dependent actomyosin ATPase activity and phosphorylation of the light chains were significantly and progressively depressed in the presence of increasing concentrations of phosphatase (0.1–0.9 U/ml). The concentration of Ca^{2+} (1.1×10^{-6} *M*) required for half-maximal activation of either ATPase activity or light-chain phosphorylation increased by 70% in the presence of 0.1 U phosphatase/ml. Neither the maximal rate of Ca^{2+}-sensitive ATP hydrolysis (39 ± 0.8 nmole/min/mg actomyosin) nor the extent of phosphorylation (0.68 ± 0.05 mole PO_4/mole light chain) was altered at $>5 \times 10^{-6}$ *M* Ca^{2+}. ATPase activity was correlated to light-chain phosphorylation under diverse conditions including the presence or absence of 1 μM calmodulin, different concentrations of phosphatase (0–0.9 U/ml), and different concentrations of Ca^{2+} (10^{-8} to 1.25×10^{-5} *M*). However, significant phosphorylation was present (20–25% of maximum) in the absence of Ca^{2+}-dependent ATPase activity and only 15% of the maximal rate of ATP hydrolysis was expressed until phosphorylation attained 50% of its maximal value. These findings are consistent with the ordered model of myosin phosphorylation suggested by A. Persechini and D. J. Hartshorne [*Science (Washington, DC)*,**213**:1383–285, 1961] (36). They also suggest that myosin phosphatase may participate in modulating actin–myosin interactions in vascular smooth muscle.

A large body of evidence suggests that the Ca^{2+}-regulatory mechanism for actin–myosin interaction in smooth muscle involves phosphorylation of the myosin light chains [for reviews see (1, 2)]. Information regarding functional consequences of light-chain phosphorylation by the Ca^{2+}-dependent myosin light-chain kinase has accumulated rapidly. In contrast, relatively little is known of the functional consequences of phosphatases which dephosphorylate phosphorylated myosin (3).

Pato and Adelstein, working with the smooth muscle of turkey gizzard, identified several myosin light-chain phosphatases. Phosphatase I, an oligomeric enzyme consisting of three subunits, was more effective in dephosphorylating light-chain kinase than it was in dephosphorylating free, or isolated, myosin light chains (4). Phosphatase II, which consisted of a single subunit, was more effective in dephosphorylating free light chains (5). However, each of these enzymes showed low phosphatase activity against phosphorylated light chains in native myosin. In contrast, phosphatase III, an enzyme of unknown structure, was more active when phosphorylated native myosin was used as substrate than when free light chains were used as substrate (6). More recently, Werth *et al.* (7) described an aortic cation-dependent phosphatase consisting of two subunits which showed high enzymic activity with respect to dephosphorylation of native myosin. The effects of this phosphatase on actin–myosin interaction were not assessed. The existence of these multiple enzyme forms expressing different substrate specificities exemplifies some of the well-recognized complexities associated with characterization and purifica-

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tion of protein phosphatases (3, 8–10). They also underscore the need for employing appropriate substrates (i.e., native myosin versus denatured light chains) in evaluating the physiological significance of a given phosphatase preparation.

Recent studies in our laboratory also showed that multiple forms of protein phosphatase are present in aortic vascular smooth muscle (3, 11–14). One of these was highly effective in dephosphorylating myosin light chains (12, 13). This phosphatase also accelerated relaxation of detergent-skinned arterial smooth muscle (15), dephosphorylated native myosin present in aortic actomyosin, and inhibited superprecipitation of the actomyosin (13).

In this report we show that the aortic myosin phosphatase reduces responsiveness to Ca^{2+} in skinned arterial segments. Moreover, the enzyme also reduces the Ca^{2+}-sensitivity of actin-activated myosin ATPase in native aortic actomyosin. This effect is correlated to corresponding decreases in the extent of myosin light-chain phosphorylation. These results are consistent with the hypothesis that phosphatase(s) may participate in modulating actin–myosin interaction in vascular smooth muscle.

Methods. *Preparation of skinned arterial smooth muscle.* Skinned carotid medial strips were prepared from porcine carotid arteries as described earlier (15) using a method adapted from Gordon (16). Medial strips were incubated at 0°C for 30 min in a solution containing 20 m*M* imidazole, pH 7.4, 5 m*M* ethylene glycol bis(2-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 50 m*M* KCl, and 150 m*M* sucrose followed by an additional 30 min in the same solution containing 1% Triton X-100 and 0.5 m*M* dithioerythritol (DTE). The strips were transferred to 20 m*M* imidazole, pH 7.4, 4 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* $MgCl_2$, 7.5 m*M* ATP, 1 m*M* NaN_3, 0.5 m*M* DTE, and 50% glycerol. Skinned segments stored in this solution at −20°C retained their responsiveness to Ca^{2+} for up to 2 months.

Preparation of proteins. The preparation of Ca^{2+}-dependent native actomyosin was adapted from procedures described previously (17–19). All steps were performed at 4°C. Briefly, bovine aortic muscularis (50–100 g) was homogenized in 5 vol of 100 m*M* KCl, 0.5 m*M* DTE, 0.1 m*M* phenylmethylsulfonyl fluoride, and 10 m*M* Tris, pH 7.0. The washed sediment was extracted (4 hr) in 2 vol of 20 m*M* Tris, pH 7.0, 80 m*M* KCl, 0.4 m*M* $MgCl_2$, 30 m*M* 2-mercaptoethanol, 4 m*M* EGTA, and 4 m*M* ATP. Actomyosin was precipitated by dialysis against 20 m*M* Tris, pH 7.0, 50 m*M* KCl, 4 m*M* $MgCl_2$, 30 m*M* 2-mercaptoethanol and 0.5 m*M* EGTA. The precipitated protein was sedimented (10,000 rpm/15 min), washed three times in 4 pellet vol of 20 m*M* Tris, pH 7.0, 15 m*M* 2-mercaptoethanol and 1% Triton X-100, and four times in the same solution without Triton X-100. Protein content of the final product, determined by the Lowry procedure (20), was adjusted to 4 mg/ml. Actomyosin ATPase activity retained Ca^{2+} dependence for at least 2 weeks during storage at 4°C.

Bovine testicular calmodulin was purified to homogeneity by the method of Teo *et al.* (21). Cardiac light chains were prepared from bovine myocardium (22) and phosphorylated with [γ-^{32}P]ATP and aortic myosin light-chain kinase (3, 13). As described previously (3, 11–13) the aortic phosphatase was isolated by procedures involving ion-exchange chromatography on DEAE Sephadex A-50, precipitation with ammonium sulfate, gel filtration on Ultrogel AcA-34 (LKB), and two cycles of chromatography on polylysine-Sepharose. The specific activity of the enzyme was 1600 U/mg protein when phosphorylated cardiac myosin light chains (4 μM) were used as substrate. One unit (U) of phosphatase activity is that amount of enzyme which releases 1 nmole ^{32}P/min at 30°C from phosphorylated light chains. In accord with studies cited earlier, the aortic phosphatase was active in the absence of either Mg^{2+}, Mn^{2+}, or Co^{2+} and was free of calmodulin, calmodulin-binding proteins, myosin light-chain kinase, and cAMP-dependent protein kinase.

Studies with skinned arterial smooth muscle. Skinned fiber bundles (5–7 mm long, 100–200 μm thick) were mounted horizontally with a cellulose-based glue between a glass post attached to an isometric force transducer (Statham, UC2) and a short glass rod fixed to a micrometer drive (15). The bundle was stretched by about 5% to a resting

tension of 0.1–0.3 mN and bathed in 250 μl of a "relaxing solution" containing 20 m*M* Tris, pH 6.7, 4 m*M* EGTA, 7.5 m*M* ATP, 10 m*M* $MgCl_2$, 1 m*M* NaN_3, 2 m*M* DTE, 0.05 μ*M* calmodulin, and an ATP regenerating system consisting of 10 m*M* phosphocreatine and 10 U/ml of creatine phosphokinase. The concentration of Ca^{2+} was $<10^{-8}$ *M* and the temperature of the solution was maintained at 25°C by means of a circulating water pump. Contraction was elicited by transferring the fiber to a "contracting solution" of similar composition wherein EGTA was partially replaced with Ca-EGTA to provide a given concentration of Ca^{2+}. The concentration of Ca^{2+} was adjusted by using an apparent dissociation constant of 1.6×10^{-6} *M* for EGTA at pH 6.7 (23). This constant was corrected for the fact that pH refers to H^+ activity rather than concentration (24).

Three indices were used to assess the influence of aortic phosphatase on actin–myosin interaction: (a) rate of isometric force development (*dF/dt*) at a submaximal concentration of 6.8×10^{-7} *M* Ca^{2+}, (b) responsiveness or sensitivity of the fibers to Ca^{2+} as reflected by the ratio of isometric force attained at the same submaximal concentration of Ca^{2+} to force attained during maximal stimulation with 1.6×10^{-6} *M* Ca^{2+}, and (c) $t_{1/2}$ for relaxation occurring when the concentration of Ca^{2+} was reduced to $<10^{-8}$ *M* (4 m*M* EGTA without added Ca^{2+}).

Accordingly, test fibers ($n = 7$) were contracted in 6.8×10^{-7} *M* Ca^{2+} until isometric force attained a steady level (5–8 min) and then in 1.6×10^{-6} *M* Ca^{2+} until maximal isometric force developed (8–12 min). After relaxation in the absence of Ca^{2+} ($<10^{-8}$ *M*) the fiber was incubated for 45 min (25°C) in the same "relaxing solution" supplemented with aortic phosphatase (16 U/ml) and the contraction–relaxation cycle was repeated. Responses obtained in the presence of phosphatase, with respect to the three indices defined above, were expressed as a percentage of the responses obtained in the initial contraction–relaxation cycle elicited in the absence of added phosphatase. Thus, each fiber served as its own control. In addition, a different series of fibers ($n = 7$) were also subjected to a similar cycle of contraction–relaxation, then incubated for 45 min in "relaxing solution" without added phosphatase and subjected to a second cycle of contraction–relaxation. These fibers were included for study to ensure that the effects of added phosphatase observed in test fibers were not ascribable to time-dependent changes which occur during incubation of skinned fibers with low concentrations of calmodulin (25). Where appropriate, the statistical significance ($P < 0.05$) of differences between responses obtained in the presence and absence of phosphatase was evaluated with Student's *t* test.

Studies with aortic native actomyosin. The influence of added phosphatase on actin–myosin interaction was studied in terms of its effects on ATPase activity. Since actomyosin contains native myosin these preparations also permitted assessment of the influence of phosphatase on myosin phosphorylation.

Actomyosin ATPase activity was determined at 30°C in a reaction mixture (1600 μl) containing 25 m*M* Tris, pH 7.0, 4 m*M* 2-mercaptoethanol, 50 m*M* KCl, 20 m*M* $MgCl_2$, 4 m*M* EGTA, 1 mg/ml actomyosin and a specified concentration of Ca^{2+}. As in studies with skinned fibers, the desired concentration of Ca^{2+} was achieved by means of a CaEGTA buffer system. However, correcting for pH 7.0, an apparent dissociation constant of 4.2×10^{-7} *M* was used for calculations of Ca^{2+} concentration (23, 24). The ATPase reaction was started by adding ATP (1 m*M*, final concentration) to the reaction mixture. Rates of ATP hydrolysis were determined from linear time course assays under conditions where less than 20% of the substrate was hydrolyzed (18, 19, 26). At 0, 2, 4, and 6 min of incubation with ATP, 375 μl of the reaction mixture was withdrawn and added to 125 μl of 125 m*M* EDTA and 13.3% sodium dodecyl sulfate (SDS) to stop the reaction. Of this mixture 400 μl was used for determination of inorganic phosphate (27). The remainder of the mixture (100 μl) was used to determine the extent of myosin light-chain phosphorylation by isoelectric focusing on polyacrylamide gels (18, 19, 28–30).

The effects of different concentrations of phosphatase (0.1–0.9 U/ml reaction mixture)

on both ATPase activity and light-chain phosphorylation of the native myosin were assessed at a submaximal concentration of Ca^{2+} (1.25×10^{-6} *M*). This experimental design was used because of the possibility that the influences of added phosphatase might be blunted at concentrations of Ca^{2+} promoting maximal interaction between actin and myosin. To test this possibility, we also studied the effects of a fixed concentration of phosphatase (0.1 U/ml reaction mixture) in the presence of different concentrations of Ca^{2+} ($<10^{-8}$ to 1.25×10^{-5} *M*). The relationship between extent of myosin light-chain phosphorylation and extent of actin–myosin interaction (ATPase activity) was studied in all experiments.

Results. *Studies with skinned porcine carotid arterial segments.* Contractile responses in detergent-skinned fiber bundles were modified in three ways following incubation in the presence of added phosphatase (Figs. 1 and 2). First, the *dF/dt* was decreased. Second, their responsiveness or sensitivity to Ca^{2+} (force in low $[Ca^{2+}]$/force in high $[Ca^{2+}]$) was also decreased. Third, the $t_{1/2}$ for relaxation was significantly reduced. Four of seven fiber bundles studied responded in a manner similar to the pattern shown in Fig. 1. That is, the effects of added phosphatase were reversible in that the reduced levels of *dF/dt*,

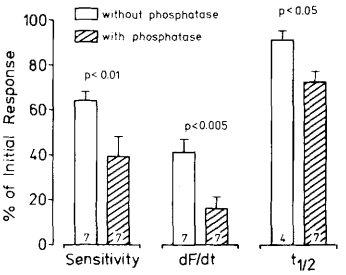

FIG. 2. The influence of aortic phosphatase (16 U/ml) on responsiveness (sensitivity) of skinned fiber bundles to Ca^{2+}, *dF/dt* in 0.68 μM Ca^{2+}, and $t_{1/2}$ for relaxation. One series of fibers (clear bars) was contracted sequentially in 0.68 μM Ca^{2+}, and 1.6 μM Ca^{2+}, and then relaxed by lowering Ca^{2+} to $<10^{-8}$ *M*. The fibers were incubated in relaxing solution for 45 min as described under Methods, and the contraction–relaxation sequence was repeated. Responses obtained in this second sequence were expressed as a percentage of the initial responses in the first contraction–relaxation (see Fig. 1). In a different series of fiber bundles studied (striped bars), phosphatase was included in the relaxing solution during the entire 45-min incubation before eliciting the second contraction–relaxation sequence. Thus, each series of fibers served as its own control. Numbers in each bar show the number of fibers studied and small vertical bars represent 1 SEM. Since values for initial responses were similar in both series of fibers they were grouped together. Initial values (n = 4) for sensitivity, *dF/dt*, and $t_{1/2}$ were respectively 0.43 ± 0.11, 0.21 ± 0.09 mN/min, and 3.9 ± 0.4 min. Maximal isometric force attained in 1.6 μM Ca^{2+} was 1.74 ± 0.22 mN, whereas force attained in 0.68 μM Ca^{2+} was 0.76 ± 0.14 mN.

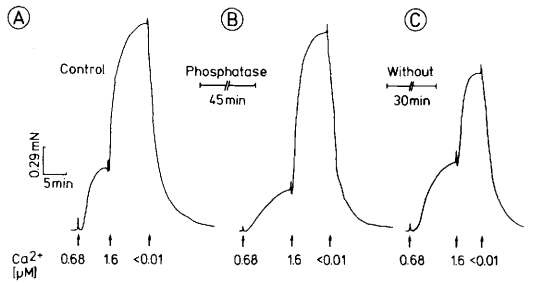

FIG. 1. Influence of aortic phosphatase on contraction and relaxation of a skinned fiber bundle from porcine carotid artery. Relative to control responses (A), the rate of isometric force development in low Ca^{2+}, responsiveness to Ca^{2+} (ratio of isometric force attained in low Ca^{2+} to force attained in high Ca^{2+}), and time for 50% relaxation ($t_{1/2}$) were all decreased after incubation with phosphatase, 16 U/ml (B). However, following continued incubation in phosphatase-free medium (C), *dF/dt*, responsiveness to Ca^{2+}, and relaxation time recovered to near control values.

responsiveness to Ca^{2+}, and $t_{1/2}$ for relaxation increased toward control levels during the test contraction elicited 30 min after the enzyme-treated fiber bundles were incubated in the absence of added phosphatase. In the remaining three preparations return of control responsiveness did not occur.

As in earlier studies (15, 25) some depression of mechanical responsiveness was always observed in a second contraction sequence induced in bundles that were studied in the absence of phosphatase. However, such depression was invariably more pronounced in the presence of added phosphatase. For example, in seven control fibers studied, *dF/dt* in response to 6.8×10^{-7} *M* Ca^{2+} decreased by about 55% during a second contraction elicited after 45 min of incubation in the absence of phosphatase (Fig. 2). Nevertheless, the decrease in *dF/dt* was markedly (85%)

and significantly ($P < 0.005$) more pronounced in seven different bundles incubated with phosphatase for the same period of time (see Methods).

Studies with native actomyosin from bovine aortic smooth muscle. Four different preparations of Ca^{2+}-sensitive aortic actomyosin were studied. Actomyosin ATPase activity, an index of actin–myosin interaction, was 47.1 ± 0.8 nmole ATP hydrolyzed/min/mg in the presence of 1.25×10^{-5} *M* Ca^{2+} and only 8.4 ± 0.9 nmole/min/mg in the absence of Ca^{2+} (4 m*M* EGTA). Both actomyosin ATPase activity and extent of myosin light-chain phosphorylation were markedly depressed in reaction mixtures containing exogenous phosphatase (Figs. 3–5). Such depression was also evident even when calmodulin (1 μM) was added to mixtures containing a submaximal concentration of Ca^{2+} (1.25×10^{-6} *M*). Thus, although added calmodulin enhanced Ca^{2+}-sensitive ATPase activity and light-chain phosphorylation (Fig. 3A, columns a and c), the extent of stimulation was blunted by the phosphatase (Fig. 3A, columns a and d). ATPase activity manifest in the different reaction mixtures studied was positively correlated to the level of myosin light-chain phosphorylation (Fig. 3B). However, it is noteworthy that essentially no Ca^{2+}-sensitive ATPase activity was demonstrable even though light-chain phosphorylation was evident to the extent of 20–25%.

The inhibitory influence of phosphatase on both actin–myosin interaction (ATPase activity) and myosin light-chain phosphorylation was dependent on the final concentration of added phosphatase (Fig. 4). In the presence of the submaximal concentration of Ca^{2+} tested (1.25×10^{-6} *M*), actomyosin ATPase activity and extent of light-chain phosphorylation progressively decreased as the concentration of phosphatase was increased. Moreover, within the range of phosphatase concentrations tested (0.1–0.9 U/ml), actomyosin ATPase activity was closely related to the degree of myosin light-chain phosphorylation (Fig. 4B). However, in accordance with the results shown in Fig. 3B, although Ca^{2+}-sensitive ATPase activity was virtually abolished at the highest concentration of phosphatase tested the light chains remained phosphorylated to the extent of about 20% (Fig. 4B).

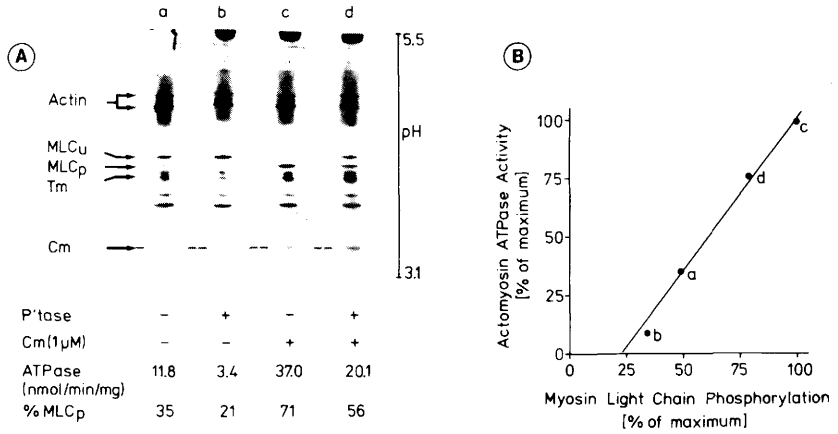

FIG. 3. Panel A shows IEF gels of an actomyosin preparation (1 mg/ml; 94 μg of actomyosin/gel) incubated with 1.25×10^{-6} *M* Ca^{2+} in the presence and absence of added phosphatase (0.1 U/ml) and/or 1 μM calmodulin. Arrows at left identify actin, unphosphorylated form of regulatory myosin light-chain (MLC_u), phosphorylated light chain (MLCp), tropomyosin (T_m), and added calmodulin (Cm). The pH gradient is given at right. Reaction conditions, Ca^{2+}-dependent actomyosin ATPase activities (nmole ATP hydrolyzed/min/mg actomyosin in 1.25×10^{-6} *M* Ca^{2+} minus activity at $<10^{-8}$ *M* Ca^{2+}), and extent of light-chain phosphorylation are listed below the gels. Basal ATPase activity in the absence of Ca^{2+} ($<10^{-8}$ *M*) was 7.6 nmole/min/mg. The relationship between Ca^{2+}-dependent ATPase activity and MLC phosphorylation as determined by densitometry of gels a, b, c, and d, is shown in Panel B (see Methods and Results for details).

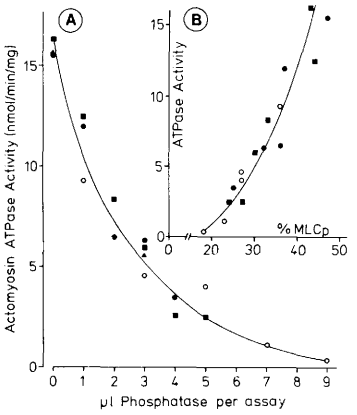

FIG. 4. Panel A shows that increasing concentrations of added phosphatase resulted in progressively more pronounced inhibition of Ca^{2+}-dependent actomyosin ATPase activity. Four different preparations, each identified by a different symbol, were studied in the presence of a submaximal concentration of Ca^{2+} (1.25×10^{-6} M). Basal ATPase activity measured with $<10^{-8}$ M Ca^{2+} was 8.4 ± 0.9 nmole ATP hydrolyzed/min/mg. The final concentration of added phosphatase in the reaction mixtures was 0.1 to 0.9 U/ml. Panel B shows the relationship between ATPase activity and MLC phosphorylation in three of the preparations studied.

Based on the data given in Fig. 4, a single concentration of phosphatase (0.1 U/ml) was selected to study its effects on relationships among concentration of Ca^{2+}, actomyosin ATPase activity, and myosin light-chain phosphorylation. The sigmoidal relationship between ATPase activity and concentration of Ca^{2+} was shifted to the right in parallel fashion when the spontaneously active phosphatase was included in the reaction mixtures (Fig. 5A). Accordingly, at high concentrations of Ca^{2+} ($\geq 5 \times 10^{-6}$ M) ATPase activity was the same in the presence and absence of phosphatase. In contrast, ATPase activity was markedly depressed in the presence of phosphatase at lower intermediate concentrations of Ca^{2+}. For example, at 1.25×10^{-6} M Ca^{2+} ATPase activity determined in the presence of phosphatase (7 nmole ATP hydrolyzed/min/mg) was decreased by 70% relative to the activity expressed in the absence of phosphatase (23 nmole ATP hydrolyzed/min/mg). Similarly, the concentration of Ca^{2+} required for half maximal activation ($A_{0.5}$) of actomyosin ATPase in the absence of phosphatase (1.1×10^{-6} M Ca^{2+}) was increased by about 65% (1.7×10^{-6} M Ca^{2+}) in the presence of phosphatase.

These phosphatase-mediated changes in Ca^{2+}-dependent ATPase activity were associated with parallel changes in Ca^{2+}-dependent phosphorylation of the myosin light chains (Fig. 5B). The Ca^{2+} $A_{0.5}$ for light-chain phosphorylation in the absence of added phosphatase (1.08×10^{-6} M) was virtually identical to the $A_{0.5}$ for activation of actomyosin ATPase (Fig. 4B). Similarly, the marked increase in $A_{0.5}$ in the presence of phosphatase (1.66×10^{-6} M) was essentially the same for both phosphorylation of the light chains and activation of actomyosin ATPase activity.

Fig. 6 shows the normalized relationship between Ca^{2+}-sensitive actomyosin ATPase activity and myosin light-chain phosphorylation for data obtained with different concentrations of Ca^{2+}, and in the presence or absence of either phosphatase or calmodulin. In spite of the diverse experimental conditions studied, the extent of actin–myosin interaction was tightly coupled to the extent of light-chain phosphorylation. Three additional points merit comment. First, the sigmoidal

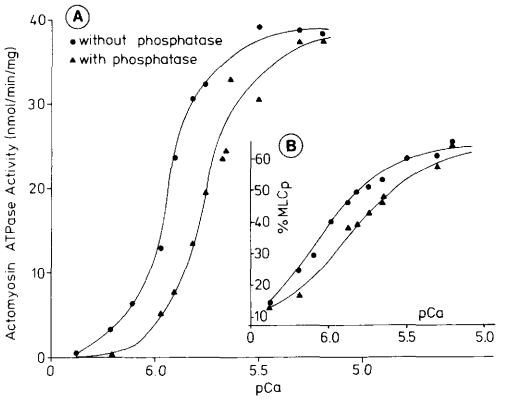

FIG. 5. The relationship between Ca^{2+}-dependent actomyosin ATPase activity and concentration of Ca^{2+} (pCa = $-\log[Ca^{2+}]$) is shown in Panel A for assays performed in the presence (▲, 0.1 U/ml) and absence (●) of added phosphatase. Basal ATPase activity (Ca^{2+} $< 10^{-8}$ M) was 8.9 nmole/min/mg. Each point is the mean of determinations in triplicate. Panel B shows the relationship between extent of light chain phosphorylation (determined by IEF of aliquots from the same reaction mixtures) and pCa.

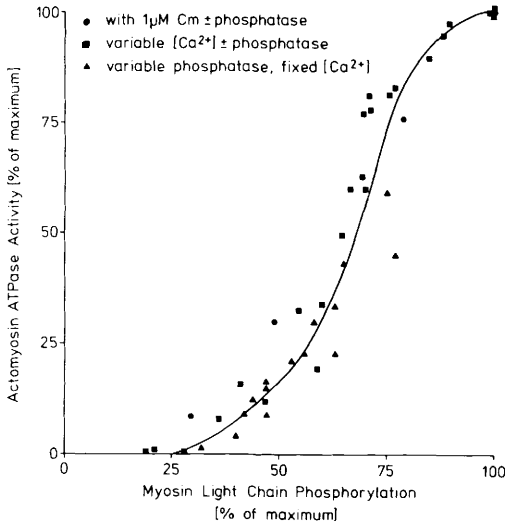

FIG. 6. Relationship between Ca^{2+}-dependent actomyosin ATPase activity and extent of light-chain phosphorylation during different experimental conditions is represented by the symbols indicated. Maximal Ca^{2+}-dependent ATPase activity was 39 ± 0.8 nmole/min/mg (n = 4) and maximal phosphorylation was 0.68 ± 0.05 mole PO_4/mole MLC (n = 4).

curve shows that a significant degree of light-chain phosphorylation (25%) was present in the absence of any apparent actin–myosin interaction. Second, relatively little Ca^{2+}-sensitive actomyosin ATPase activity was expressed (i.e., <15% of maximum) until light-chain phosphorylation attained 50% of its maximal value. Third, ATPase activity increased markedly when the extent of phosphorylation exceeded 50%.

Discussion. The results of this study show that a myosin light-chain phosphatase prepared from bovine aortic smooth muscle markedly depresses Ca^{2+}-dependent actin–myosin interaction in both skinned arterial segments (Figs. 1 and 2) and aortic native actomyosin (Figs. 3–6). Phosphatase mediated inhibitory effects in skinned preparations were reflected by depression of dF/dt in submaximal Ca^{2+} (6.8×10^{-7} M), reduced responsiveness to Ca^{2+}, and decreased relaxation time. Similarly, inhibitory effects in actomyosin were reflected by decreased phosphorylation of the regulatory myosin light chains, reduced ATPase activity, and decreased sensitivity to Ca^{2+}. These findings suggest that phosphatase which is effective in dephosphorylating phosphorylated myosin may participate in modulating actin–myosin interaction in vascular smooth muscle.

Since the enzymic activity of the phosphatase used in this study is independent of divalent cations (12, 13), it is different from the Mg^{2+}-dependent phosphatase identified in turkey gizzard (5) or the Mn^{2+}/Co^{2+}-dependent aortic phosphatase (7). Moreover, the spontaneously active phosphatase which we used dephosphorylates native myosin thereby distinguishing it from phosphatase I of turkey gizzard (4). Our enzyme may be related to the three-subunit gizzard phosphatase described by Onishi *et al.* (31), or to gizzard phosphatase III (6), both of which are effective in dephosphorylating native myosin. However, since the subunit structure is unknown for either the aortic spontaneously active phosphatase used in this study or gizzard phosphatase III, further studies are required to test this possibility.

Because the molecular weight of the aortic phosphatase which we studied is greater than 250,000 a high concentration of phosphatase (16 U/ml) and a 45-min period of incubation was employed to permit diffusion of the enzyme into the skinned fiber bundle (Fig. 1). The demonstration that its inhibitory effects were reversible in four of seven fibers studied may be due to limited diffusion of the enzyme out of the fiber bundle when transferred to phosphatase-free medium.

The structural and functional integrity of myosin in the actomyosin preparations studied is shown by the high level of Ca^{2+}-dependent ATPase activity (39 nmole ATP/min/mg) which is expressed as the difference in activity measured with (47 nmole ATP/min/mg) and without (8 nmole ATP/min/mg) excess Ca^{2+} (1.5×10^{-5} M Ca^{2+}). However, stimulation of ATPase activity and light-chain phosphorylation by added calmodulin indicates that the actomyosin probably contained limiting amounts of copurifying calmodulin.

Phosphatase mediated inhibition of actin–myosin interaction in both skinned fiber bundles and native actomyosin can be interpreted within the framework of the hypothesis that the effects were ascribable to enhanced dephosphorylation of phosphorylated light chains. Although assessment of the extent of

light-chain phosphorylation in skinned fibers was not attempted in this study, previous studies have shown that Ca^{2+}-dependent isometric force is correlated to light-chain phosphorylation in fibers prepared with either the same (32) or a different skinning procedure (33).

Within the framework of the stated hypothesis, the inhibition of dF/dt and isometric force observed in skinned fibers incubated with submaximal Ca^{2+} (6.87×10^{-7} M, Fig. 1B) probably reflects ongoing light-chain phosphorylation, and concurrent but enhanced dephosphorylation. However, maximal activation of endogenous myosin light-chain kinase by high concentrations of Ca^{2+} in either the skinned fiber preparations (1.6×10^{-6} M Ca^{2+}) or native acotmyosin ($\geq 5 \times 10^{-6}$ M) probably offsets the inhibitory effects of added phosphatase. Under these conditions, isometric force attained in the skinned fiber, and ATPase activity expressed by the actomyosin, would closely approximate control values obtained in the absence of added phosphatase. In other words, the inhibitory effects of the phosphatase on actin–myosin interaction would be inversely related to the ratio of kinase to phosphatase activity. Accordingly, immersion of maximally contracted fibers into Ca^{2+}-free medium ($<10^{-8}$ M Ca^{2+}) would be associated with inactivation of the kinase and a consequent decrease in the kinase to phosphatase activity ratio. This situation probably causes more rapid dephosphorylation of the light chains and accelerated relaxation.

It should be recognized that the concentration of Ca^{2+} required for maximal contraction of skinned fiber bundles (1.6×10^{-6} M) was lower than the concentration required for maximal stimulation of actomyosin ATPase activity ($\geq 5 \times 10^{-6}$ M Ca^{2+}). This may be due, at least in part, to the inclusion of calmodulin in the fiber bathing medium and its omission from the actomyosin preparations used to study Ca^{2+} dependency.

It is important to emphasize that concomitant phosphatase-mediated inhibition of light-chain phosphorylation and actomyosin ATPase activity occurred under diverse experimental conditions. These included studies performed in the presence and absence of added calmodulin (Fig. 3), increasing concentrations of phosphatase (Fig. 4) and increasing concentrations of Ca^{2+} (Fig. 5). It is likely that the extent of phosphatase-mediated inhibition of actomyosin ATPase activity is underestimated because concomitant phosphorylation and dephosphorylation are probably associated with apparent pseudo-ATPase activity. That is, analysis of actomyosin reaction mixtures for inorganic phosphate released from ATP measured both actin-activated myosin ATPase activity per se, and the apparent pseudo-ATPase activity associated with concurrent phosphorylation and dephosphorylation of the myosin light chains. The magnitude of the pseudo-ATPase activity operative in the present studies is unknown, but available data suggest that it is small relative to the actomyosin ATPase activity (34, 35). Nevertheless, the positive correlation between extent of light chain phosphorylation and magnitude of actomyosin ATPase activity shown under the different conditions studied strongly supports the hypothesis that the inhibitory effects of the aortic phosphatase are ascribable to enhanced dephosphorylation of the light chains (Fig. 6).

The present findings also show that a significant degree of light-chain phosphorylation (20–25% of maximal phosphorylation) exists in the absence of significant Ca^{2+}-sensitive actomyosin ATPase activity (Figs. 3–6). This may be related to the ordered or sequential model for light-chain phosphorylation in native myosin developed by Persechini and Hartshorne (36, 37). In this model, phosphorylation of one of the phosphorylatable light chains must occur before phosphorylation of the second light chain occurs. Their model also suggests that both light chains must be phosphorylated in order to promote actin–myosin interaction. Accordingly, little or no expression of ATPase would be demonstrable until about 50% of the light chains were phosphorylated. The data in Fig. 6 show that only about 15% of the maximal Ca^{2+}-sensitive actomyosin ATPase activity attainable was expressed before 50% of the maximal level of phosphorylation was attained. Thus, the relationship between light-chain phosphorylation and actin–myosin interaction observed in aortic native actomyosin in either the presence or absence of added phosphatase is, to a first approximation,

compatible with the Persichini–Hartshorne model (36, 37). Interestingly, the maximal level of phosphorylation attained in the present studies with actomyosin (63–71%, Figs. 3–5) is comparable to levels of light-chain phosphorylation attained during maximal stimulation of living carotid arterial smooth muscle (29, 38) or skinned preparations from the same artery (32, 39).

The results of this study do not preclude the possibility that mechanisms in addition to phosphorylation of the light chains may also be involved in the Ca^{2+}-regulatory mechanism(s) for actin–myosin interaction in smooth muscle. Conceivably, other regulatory proteins such as those possibly involved in potential actin-linked mechanisms (2) may have been lost during the preparation of either skinned fiber bundles or native actomyosin.

Nevertheless, this study shows that an aortic myosin phosphatase markedly influences Ca^{2+}-dependent actin–myosin interaction in two different models for contraction in vascular smooth muscle. In this context, it is becoming increasingly clear that protein phosphatases are regulated enzymes (3, 8–10, 40). Conceivably, regulation of myosin phosphatase activity may participate in modulating contraction and relaxation of vascular smooth muscle.

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Note added in proof. Since submission of this manuscript for publication, we have found that the phosphatase used in the studies reported was subject to modulation *in vitro* by polycationic effectors such as lysine-rich histone-H_1 and polylysine. Thus, the phosphatase is virtually identical to the enzyme which we described in Ref. (40). That is, low concentrations of polylysine (mol wt = 13,000; 2–5 μg/ml) stimulated expressed phosphorylase phosphatase activity 6- to 10-fold, but markedly inhibited dephosphorylation of myocardial myosin light chains. We would like to suggest that this enzyme be referred to as PCM-phosphatase (polycation-modulable).

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