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Relationships between Ca^{2+}, Myosin Light Chains, and ATPase in Bovine Aortic Actomyosin: Presence of Ca^{2+}-Requiring Inactivation Factor (40677)[1]

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The important role of Ca^{2+} in regulating contractile activity in vascular smooth muscle is well recognized (1, 2). However, the mechanistic basis for this regulation has not been completely elucidated. Studies with contractile proteins from several different smooth muscles including avian gizzard (3–5), porcine stomach (6), and hamster vas deferens (7) suggest that the Ca^{2+} regulatory mechanism is linked to the thick myosin filament and involves Ca^{2+}-dependent phosphorylation of the 15,000- to 20,000-dalton myosin light chains. This contrasts with striated muscle where the regulatory mechanism is linked to the Ca^{2+}-receptive protein troponin located on the thin actin filament (8). Nevertheless, Mikawa *et al.* (9) claim that Ca^{2+} regulation in smooth muscle is independent of phosphorylation and is associated with an 80,000-dalton troponin-like moiety.

Recently, we reported that Ca^{2+}-dependent phosphorylation of the 15,500-dalton myosin light chains occurs in native actomyosin from bovine aorta (10). The K_m for Ca^{2+} (3×10^{-7} *M*) in this reaction was the same as the K_m for Ca^{2+} in activating aortic actomyosin ATPase. This suggests that the Ca^{2+} regulatory mechanism for actin–myosin interactions in vascular smooth muscle may also involve Ca^{2+}-dependent phosphorylation of the myosin light chains. The hypothesis is particularly attractive because myosin-linked regulation is present in vascular smooth muscle (11, 12).

Ca^{2+} regulation is rapidly lost in actomyosin from vascular smooth muscle (2). Whether or not this loss of Ca^{2+} sensitivity is associated with changes in the phosphorylatable light chains is unknown. In this context, it is important to recognize that in other tissues Ca^{2+} has also been shown to activate proteases which specifically degrade proteins of the contractile apparatus. A Ca^{2+}-stimulated protease which degrades α-actinin and the Z line of myofibrils has been described in skeletal muscle (13). In cardiac muscle, an apparently different Ca^{2+}-stimulated protease exists which degrades only the phosphorylatable light chain (14). It is therefore possible that actomyosin from vascular smooth muscle also contains a Ca^{2+}-dependent factor which degrades or inactivates proteins involved in regulating actin–myosin interactions.

In this study we have examined phosphorylation of aortic actomyosin in preparations which required Ca^{2+} for hydrolysis of ATP and in stored preparations which no longer required Ca^{2+} for hydrolysis of ATP. Emphasis was placed on determining (a) if the loss of Ca^{2+} regulation was associated with changes in the phosphorylatable light chains, and (b) whether or not the actomyosin contained a Ca^{2+}-stimulated factor which could inactivate the regulatory mechanism for actin–myosin interaction.

Materials and methods. The methods used for (a) preparation of bovine aortic actomyosin, (b) performance of sodium dodecyl sulfate (SDS) electrophoresis and isoelectric focusing, (c) determinations of actomyosin ATPase activity, and (d) detection of phosphorylation of the myosin light chains using [γ-^{32}P]ATP were described previously (10).

Superprecipitation of actomyosin was studied according to methods adapted from Ebashi (15). Briefly, a reaction mixture was prepared consisting of 18 m*M* morpholinopropanesulfonic acid pH 7.0 (MOPS), 10 m*M* $MgCl_2$, 10^{-5} *M* $CaCl_2$, 2 mg actomyosin/ml, and sufficient KCl to maintain ionic strength at 0.1. The mixture (2 ml) was transferred to a cuvette in a Gilford spectrophotometer which permitted continuous stirring of the cuvette contents and maintained temperature at 25°. The reaction was started by injecting

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10 μl of 200 m*M* Na_2ATP into the mixture so that its final concentration was 1 m*M*. Changes in absorbance were recorded at 660 nm. After 5 min of incubation 250 μl was withdrawn for SDS electrophoresis and isoelectric focusing. Similar mixtures containing 1 m*M* ethyleneglycol-bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) in place of $CaCl_2$ were used to assess Ca^{2+} sensitivity of the preparation.

Temporal relationships between phosphorylation of the myosin light chains and hydrolysis of ATP by actomyosin ATPase were assessed using reaction mixtures containing 0.4 to 0.6 mg actomyosin/ml and 10 μCi [γ-^{32}P]ATP/ml (Amersham, specific activity 2.2 Ci/mmole). The total concentration of ATP in the reaction mixture was 1 m*M* so that its specific activity was 2.2×10^4 cpm/nmole. At appropriate intervals aliquots of the mixture were withdrawn for determination of inorganic phosphate (ATPase activity) and phosphorylation of the myosin light chains (10).

Unless otherwise indicated, all of the above experiments were performed within 24 hr of the isolation of native actomyosin. Preliminary experiments showed that storage of the preparations at 4° resulted in time-dependent loss of Ca^{2+} sensitivity. Progressive loss of Ca^{2+} sensitivity was determined by changes in ATPase activity in the presence and absence of Ca^{2+}, superprecipitation, and analysis of electrophoretic profiles. The same actomyosin preparations were also stored in the presence of 5 m*M* EGTA to test for the presence of a Ca^{2+}-dependent factor which contributed to the loss of Ca^{2+} requirement for activation of actomyosin ATPase.

Results and discussion. Twelve preparations of actomyosin were studied. The yield of actomyosin was 2.8 ± 0.3 mg protein per gram of aortic muscularis.

In freshly prepared actomyosin, the Mg^{2+}-stimulated hydrolysis of ATP was Ca^{2+} dependent in all 12 preparations. The specific ATPase activity of fresh preparations decreased by 90% when the Ca^{2+} in the reaction mixture was chelated with 1 m*M* EGTA (Table I). In accord with earlier findings (10) isoelectric focusing (IEF) of reaction mixtures containing Ca^{2+} and ATP showed that phosphorylation of the myosin light chains occurred as evidenced by the progressive disappearance of the unphosphorylated light chain focusing at pH 5.05 and the appearance of a new phosphorylated light chain band focusing at pH 4.97. This change appears to specifically require ATP because it did not occur when ADP, AMP, cAMP, or sodium pyrophosphate was substituted for ATP (Fig. 1). We have previously shown that the change in IEF is Ca^{2+} dependent and associated with incorporation of ^{32}P from [γ-^{32}P]ATP into the new band at pH 4.97. The phosphorylation is limited to the 15,500-dalton myosin light chains (10). Our procedure has been adapted to detect phosphorylated light chains in intact vascular smooth muscle (16).

Results obtained during concomitant measurements of the time course for phosphorylation of the light chains and activation of the Mg^{2+}-stimulated actomyosin ATPase showed that light-chain phosphorylation preceded the release of inorganic phosphate (P_i) from

TABLE I. INFLUENCE OF CA^{2+} AND EGTA ON Mg^{2+}-STIMULATED ACTOMYOSIN ATPase ACTIVITY OF Ca^{2+}-SENSITIVE AND Ca^{2+}-INSENSITIVE PREPARATIONS OF AORTIC ACTOMYOSIN

Preparation	Actomyosin ATPase activity[a] (nmoles P_i/mg/min)		Ca^{2+} Sensitivity (activity in 10^{-5} *M* Ca^{2+}/ activity in 10^{-5} *M* EGTA)	*P*[b]
	In 10^{-5} *M* Ca^{2+}	In 10^{-3} *M* EGTA		
Ca^{2+}-Sensitive	32.4 ± 2.6	2.6 ± 1.9	12.5	<0.001
Ca^{2+}-Insensitive	18.3 ± 2.1	15.4 ± 1.9	1.2	>0.1 (NS)[d]
P[c]	<0.005	<0.005		

[a] Values for ATPase specific activity are expressed as means ± 1 SE for determinations on 12 different preparations of actomyosin. Each preparation was assayed within 24 h of preparation (Ca^{2+}-sensitive) and again after storage for 5 to 7 days at 4°C (Ca^{2+}-insensitive).

[b] Shows *P* value for difference in specific activity measured in the presence of Ca^{2+} and in the presence of EGTA.

[c] Shows *P* value for difference in specific activity of Ca^{2+}-sensitive and Ca^{2+}-insensitive preparations.

[d] NS, Not significantly different.

activation of the actomyosin ATPase (Fig. 2). For example, 50% of the maximal amount of phosphorylation (^{32}P incorporated) occurred after only 30 sec of incubation at a time when virtually no P_i release from ATP was yet detected. Similarly, of the total amount of P_i released during the entire 30-min period of the assay, only 7% (60 nmol P_i) was detected within 2 min after the reaction was initiated. In sharp contrast, 92% of the ^{32}P incorporated into the new phosphorylated myosin light chain band was found in the same interval. These findings, obtained with preparations from mammalian vascular smooth muscle, are in agreement with results obtained from avian gizzard actomyosin (17). The results suggest that phosphorylation occurs prior to actin-mediated activation of the Mg^{2+}-stimulated myosin ATPase. We have calculated[2] that only about 15% of the phosphorylatable light chains are actually phosphorylated under the optimal conditions of Fig. 2 (i.e., after 5 min of incubation). Because this is a native actomyosin preparation rather than a reconstituted system of purified proteins, it is probable that there is a phosphatase activity present as well as the kinase activity we are measuring. The value of 15% phosphorylation is, therefore, likely to represent a dynamic equilibrium between phosphorylation and dephosphorylation of the light chain. Additionally, it should also be noted that the time course for phosphorylation of myosin light chains *in vitro* is compatible with the time course for the development of maximal isometric force (2–4 min) in strips of vascular smooth muscle isolated from a variety of sources (18, 19) and the time course maximal vasoconstriction in several different vascular beds *in vivo* (20, 21).

All of the preparations became Ca^{2+} insen-

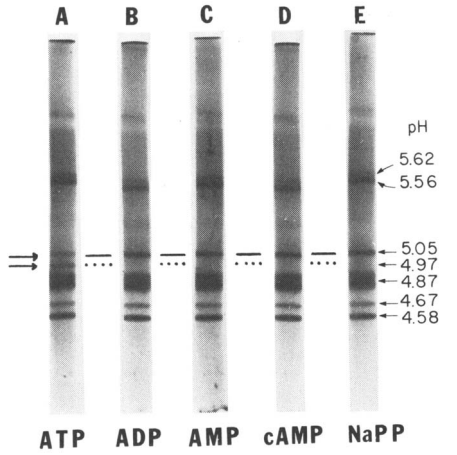

FIG. 1. Comparison of IEF of reaction mixtures containing equimolar (1 m*M*) concentrations of either ATP (A), ADP (B), AMP (C), cAMP (D), or sodium pyrophosphate (E). Each mixture contained 10^{-5} *M* $CaCl_2$. The gels were stained with Coomassie blue and each band can be located by the pH scale at right. Bands at pH 5.62–5.56 correspond to actin polymorphs, tropomyosin is in the diffuse area at pH 4.87, and the bands at pH 4.67 and 4.58 are unidentified. The unphosphorylated myosin light chain focuses at pH 5.05 (upper arrow at left), whereas the phosphorylated form focuses at pH 4.97 (lower arrow). The phosphorylated light chain (dotted line between gels) occurs only in the presence of ATP (A). The unphosphorylated band (solid line between gels) is intense in gels B–E.

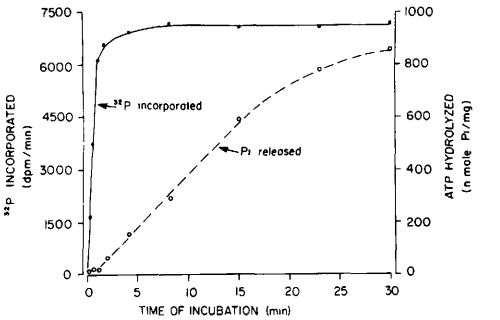

FIG. 2. Relationship between time of incubation at 25° (abscissa), incorporation of ^{32}P from [γ-^{32}P]ATP (left ordinate, solid curve), and release of inorganic phosphate (P_i) by Mg^{2+}-stimulated ATPase (right ordinate, broken curve). The incorporation of ^{32}P was determined by withdrawing aliquots from the reaction mixture at the times indicated and subjecting them to IEF and SDS electrophoresis. The myosin light-chain bands were excised and radioactivity was detected by scintillation counting (see Ref. (10)). Release of P_i was determined by ATPase assay. Phosphorylation was essentially completed before significant hydrolysis of ATP occurred by ATPase.

[2] This value for the phosporylation of the phosphorylatable light chain was obtained as follows. Given a specific activity of [γ-^{32}P]ATP = 2.2×10^4 cpm/nmol, the incorporation of a maximum of 7×10^3 dpm into the light chain (Fig. 2) represents 0.32 nmol of phosphate incorporated. The reaction mixture contained about 1 mg of protein of which at least 50% or 0.5 mg is myosin (2, 4). With a molecular weight of about 2.6×10^5, this represents 1.1 nmol of myosin. Thus, assuming two molecules of phosphorylatable light chain per myosin, there will be 2.2 nmol of light chain of which 0.32 nmol or 15% is phosphorylated.

sitive after storage at 4° for 5 to 6 days so that the specific ATPase activity was similar in the presence and absence of EGTA (Table I). The loss of Ca^{2+} sensitivity was associated with the loss of the phosphorylatable myosin light chains (Fig. 3). It is particularly noteworthy that the loss of Ca^{2+} sensitivity also was associated with an increase in the basal Mg^{2+}-stimulated ATPase activity. Thus, while the specific ATPase activity of the aged actomyosin was decreased by 50% in the presence of Ca^{2+} it was increased, relative to the fresh actomyosin, by almost sixfold in the presence of EGTA (Table I). This marked increase in basal ATPase activity measured in the presence of EGTA may be related to the concomitant loss of Ca^{2+} sensitivity and of the phosphorylatable light chains. Thus, it is possible that there are two effects of the Ca^{2+}-dependent phosphorylation of light chains. First, phosphorylation eliminates the inhibition of actin-myosin interaction imposed by the light chain in its nonphosphorylated form. Second, the presence of the phosphorylated light chain augments actin activation of myosin ATPase. Accordingly, stored preparations which have lost the light chain would show both high basal ATPase activity and little or no stimulation with Ca^{2+}. Although additional studies are required to test this hypothesis, it is interesting to note that removal of the phosphorylatable light chain from cardiac myosin enhances actin–myosin interaction (22). Interest in the cardiac findings is heightened because it is an actin-linked regulatory system.

The coincident loss of Ca^{2+} sensitivity and phosphorylatable light chains was prevented when actomyosin preparations were stored in the presence of 5 m*M* EGTA (Fig. 3). Ca^{2+}-Dependent superprecipitation of preparations stored in the absence of EGTA became progressively delayed and decreased in magnitude (Figs. 3A–D). At the same time, however, Ca^{2+}-independent superprecipitation became more pronounced. These changes in Ca^{2+} sensitivity were associated with progressive loss of the 15,500-dalton myosin light chains. In sharp contrast, preparations stored in the presence of EGTA over the same period of time retained the myosin light chains and the Ca^{2+} requirement for superprecipitation (Figs. 3E–F). Storage in EGTA invariably protected against time-dependent loss of Ca^{2+} sensitivity. Knowledge that addition of EGTA protects the biochemical integrity of aortic actomyosin preparations during storage should also prove useful to other investigators. The protective effect of EGTA is specific because no protection was afforded

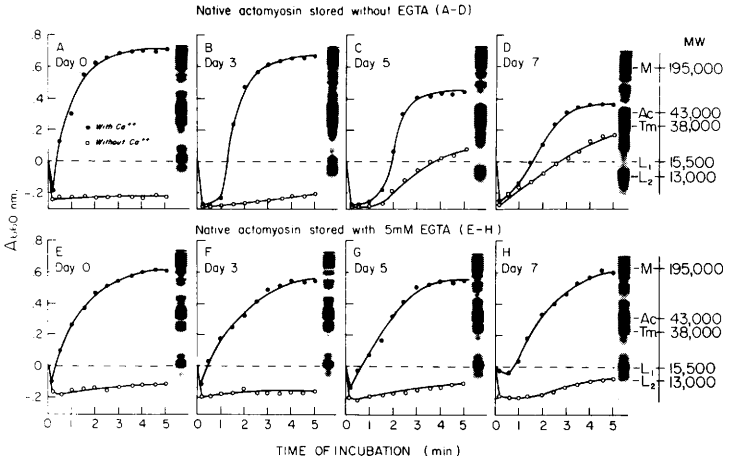

FIG. 3. Changes in Ca^{2+} sensitivity of actomyosin stored at 4° in the presence and absence of EGTA. Panels A–D show time-dependent changes in actomyosin stored for up to 7 days without EGTA. Superprecipitation was then measured turbidometrically in the presence of 10^{-5} *M* Ca^{2+} (●) or 10^{-3} *M* EGTA (○). SDS gels to the right of each panel show the changes in profiles with storage where M is myosin heavy chains, Ac is actin, Tm is tropomyosin, L_1 is the phosphorylatable light chain, and L_2 is the nonphosphorylatable light chain. Molecular weights are shown on scale at right. Panels E–F show corresponding material from the same actomyosin preparation stored in the presence of EGTA. Further details are given in the text.

when preparations were stored in the presence of soybean trypsin inhibitor, pepstatin, leupeptin, streptomyosin, chloremphenicol, sodium azide, or phenylmethylsulfonyl fluoride. The findings suggest that the aortic native actomyosin contains a Ca^{2+}-dependent factor, perhaps a Ca^{2+}-dependent protease, which digests the phosphorylatable light chain and thereby promotes the progressive loss of Ca^{2+} sensitivity. To our knowledge this is the first report of the presence of a Ca^{2+}-dependent factor in preparations from mammalian vascular smooth muscle which has high specificity for degrading the myosin light chains. Despite its relative specificity for myosin light chains, the postulated Ca^{2+}-dependent protease(s) clearly also partially degrades certain other proteins as evidenced by the disappearance of several other gel bands (e.g., 55,000 daltons) and the appearance of new bands (e.g., 80,000 daltons). Consequently, the decrease in superprecipitability as well as the alterations in Ca^{2+}-sensitive ATPase activity could also be related to modification of these other proteins. In this context, it is noteworthy that tryptic digestion of gizzard actomyosin results in concomitant loss of phosphorylatable light chains and actin-activated ATPase (4). However, partial digestion of the same preparation with papain preserves Ca^{2+} sensitivity as long as the phosphorylatable light chain remains intact. Clearly, further studies are required to characterize the Ca^{2+}-dependent factor in our preparations and define its role in maintaining function in vascular smooth muscle.

Summary. Phosphorylation of the myosin light chains occurs in Ca^{2+}-sensitive aortic actomyosin. This phosporylation specifically requires ATP and is essentially completed before significant release of P_i by actomyosin ATPase can be detected. Progressive loss of Ca^{2+} sensitivity in preparations stored at 4° is associated with progressive loss of phosphorylatable light chains so that phosphorylation no longer occurs. Although Ca^{2+}-activated ATPase is depressed in these light chain-deficient preparations, basal ATPase measured in the absence of Ca^{2+} is increased. These data suggest that the unphosphorylated light chains inhibit actin-stimulated myosin ATPase, whereas phosphorylation of the light chains relieves the inhibition and amplifies actin stimulation of myosin ATPase. During storage there is a parallel loss of the Ca^{2+} sensitivity and phosporylatable light chains. Both losses are prevented when actomyosin is stored in the presence of EGTA. These findings suggest that aortic actomyosin contains a Ca^{2+}-dependent factor, perhaps a Ca^{2+}-stimulated protease, which is responsible for progressive loss of phosporylatable light chains. The results of the present study provide further support for the hypothesis that the Ca^{2+} regulatory mechanism for actin–myosin interactions in mammalian vascular smooth muscle involves Ca^{2+}-dependent phosphorylation of the myosin light chains.

1. Bohr, D. F., Circ. Res. **32,** 665 (1973).
2. Murphy, R. A., Fed Proc. **35,** 1302 (1976).
3. Bremel, R. D. Nature (London) **252,** 405 (1974).
4. Sobieszek, A., and Small, J. V., J. Mol. Biol. **101,** 75 (1976).
5. Sherry, J. M., Górecka, A., Aksoy, M. O., Dabrowska, R., and Hartshorne, D. J., Biochemistry **17,** 4411 (1978).
6. Small, J. V., and Sobieszek, A., Eur. J. Biochem. **76,** 521 (1977).
7. Chacko, S., Conte, M. A. and Adelstein, R. S., Proc. Nat. Acad. Sci. USA **74,** 129 (1977).
8. Szent-Györgyi, A. G., Biophys. J. **15,** 707 (1975).
9. Mikawa, T., Nonomura, Y., and Ebashi, S., J. Biochem. (Tokyo) **82,** 1789 (1977).
10. DiSalvo, J., Gruenstein, E., and Silver, P., Proc. Exp. Biol. Med. **158,** 410 (1978).
11. Mrwa, U., and Rüegg, J. C., FEBS Lett. **60,** 81 (1975).
12. Frederiksen, D. W., Proc. Nat. Acad. Sci. USA **73,** 2706 (1976).
13. Dayton, W. R., Goll, D. E., Zeece, M. G., Robson, R. M., and Reville, W. J., Biochemistry **15,** 2150 (1976).
14. Bhan, A., Malhotra, A., Hatcher, V. B., Sonnenblick, E. S., and Scheuer, J., J. Mol. Cell. Cardiol. **10,** 769 (1978).
15. Ebashi, S., J. Biochem. (Tokyo) **50,** 236 (1961).
16. Driska, S. P., and Murphy, R. A., Biophys. J. **25,** 73a (1979).
17. Sobieszek, A., Eur. J. Biochem. **73,** 477 (1977).
18. DiSalvo, J., and Schmidt, C., Proc. Exp. Biol. Med. **151,** 478 (1976).
19. Bohr, D. F., Canad. Med. Ass. J. **90,** 174 (1964).
20. DiSalvo, J., and Montefusco, C. B., Amer. J. Physiol. **221,** 1576 (1971).
21. Britton, S., and DiSalvo, J., Amer. J. Physiol. **225,** 1226 (1973).
22. Malhotra, A., Huang, S., and Bahn, A., Biochemistry **18,** 461 (1979).

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