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Ca^{2+} Dependent Phosphorylation of Bovine Aortic Actomyosin[1] (40215)

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It is increasingly clear that the Ca^{2+} regulatory mechanism for actinomyosin interactions in smooth muscle differs from the mechanism which is operative in striated muscle. In striated muscle, the dominant current view suggests that Ca^{2+} interacts with troponin C, the Ca^{2+} receptive subunit of the troponin complex, thereby facilitating displacement of tropomyosin from an inhibitory position to a different position which permits interaction between actin and myosin so that force can develop (1, 2). In contrast, evidence obtained with actomyosin from a variety of smooth muscles including chicken gizzard, bovine aorta, porcine aorta and carotid artery suggests that the Ca^{2+} regulatory mechanism is not limited to the thin filament, but instead involves interactions between Ca^{2+} and myosin (3–6). Thus, the Ca^{2+} regulatory mechanism in these smooth muscles is similar to the myosin-linked system discovered in invertebrate muscles by Szent-Györgyi's group (2, 7).

The mechanistic basis for myosin-linked regulation has not been completely elucidated. However, recent studies indicate that such regulation in smooth muscles is mediated by Ca^{2+} dependent phosphorylation of the 15,000–20,000 dalton myosin light chains (8–11). Phosphorylation of these light chains has been demonstrated in arterial actomyosin (12). However, the relationship between Ca^{2+}, phosphorylation and ATPase activity in vascular smooth muscle is not known.

In this communication, we report that Ca^{2+} dependent phosphorylation occurs in bovine aortic actomyosin and that the requirement for Ca^{2+} in this reaction parallels the Ca^{2+} requirement for activation of Mg^{2+} stimulated actomyosin ATPase. We show that isoelectric focusing of actomyosin on polyacrylamide gels permits rapid and quantitative separation of phosphorylated and nonphosphorylated polypeptides.

Methods. Ca^{2+} sensitive actomyosin was prepared from bovine aortae according to methods described by Litten *et al.* (6). Briefly, the muscularis was extracted (4°) at low ionic strength (50 m*M* KCl, 4 m*M* $MgCl_2$, 2 m*M* dithiothreitol (DTT), 20 m*M* morpholinopropane sulfonic acid (MOPS pH 7.0), 2 m*M* ethylene-glycol-bis (2-aminoethyl ether)-*N,N'*-tetra acetic acid (EGTA) and 4 m*M* ATP). The actomyosin was precipitated by dialysis against low salt (25 m*M* KCl, 2 m*M* $MgCl_2$, 1 m*M* DTT, 5 m*M* MOPS pH 7.0, and 0.2 m*M* EGTA), sedimented at 10,000*g* for 15 min, washed 4 times in 8 pellet volumes of the same solution supplemented with 1% triton X-100 and 5 times with low salt solution without triton. The final product was suspended in a solution containing 18 m*M* MOPS pH 7.0, 50 m*M* KCl, 1 m*M* DTT and 1 m*M* $MgCl_2$. The concentration of protein, determined by the Lowry procedure (13), was adjusted to 10 mg/ml. Specific activity of Mg^{2+} stimulated actomyosin ATPase (nmole Pi/mg/min) was determined at 25° according to well established methods for smooth muscle actomyosin (3–6, 14). Time dependent liberation of inorganic phosphate (Pi) was determined by the method of Rockstein and Herron (15). Routinely, the reaction mixture for ATPase assays contained 18 m*M* MOPS pH 7.0, 10 m*M* $MgCl_2$, 0.8–1.0 mg actomyosin/ml, either 5×10^{-5} $CaCl_2$ or 2 m*M* EGTA, sufficient KCl to maintain ionic strength at 0.1 μm and 1 m*M'* Na_2 ATP. To examine the relationship between concentration of Ca^{2+} and ATPase activity, and between Ca^{2+} and extent of phosphorylation of CaEGTA buffer system was employed using 2×10^{-7} *M* as the apparent dissociation constant for CaEGTA at pH 7.0 (7, 16).

The technique for isoelectric focusing (IEF) of actomyosin on polyacrylamide gels was adapted from procedures described by

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Righetti and Drysdale (17). Samples of a reaction mixture prepared in the same way as for the ATPase assay and containing 100 μg actomyosin were applied to 4% polyacrylamide gels supplemented with 1% triton, 9.5 *M* Urea, and 2% ampholines (LKB: 1.6% pH 4–6, Biorad: 0.4% pH 3.5–10) such that the pH gradient which developed during electrophoresis ranged from 7.0 ± 0.15 at the top of the gel to pH 4.0 ± 0.10 at the bottom. The gels were fixed with 15% trichloroacetic acid, stained with 0.1% Coomassie blue and destained with a mixture of 10% acetic acid and 15% methanol. Densitometry of stained gels (550 nm) was performed with an Isco densitometer and the change in area of peaks showing a change in isoelectric point (change in position) was determined by weighing of carefully prepared tracings. The extent to which a given protein band was converted to a new band with a different isoelectric point was expressed as a percentage. This percent conversion was calculated from the ratio of the peak area of the band in its new position to the combined areas of the parent band and the new band on the same gel (see Results and discussion).

Sodium dodecyl sulfate (SDS) electrophoresis of actomyosin reaction mixtures was performed on 7.5% polyacrylamide gels using standard procedures described by Weber and Osborn (18). Purified protein markers for molecular weight (MW) were purchased from Pharmacia and included ribonuclease A (MW 13,700), chymotrypsinogen (MW 25,000), bovine serum albumin (MW 58,000, and aldolase (MW 13,700). Purified contractile proteins from smooth muscle were kindly supplied by Dr. R. A. Murphy (University of Virginia).

Incorporation of ^{32}P into actomyosin was studied by adding 10 μCi ATPγ^{32}P/ml of high specific activity (2.2 Ci/mmole, Amersham) so that the total concentration of ATP in the reaction mixture was essentially unchanged. Stained bands corresponding to major components of actomyosin, as well as unstained regions from the top and bottom of IEF and SDS gels, were excised and treated as described in detail by Aksoy *et al.* (8). Radioactivity was measured with a Beckman liquid scintillation counter.

Results and discussion. The Mg^{2+} stimulated hydrolysis of ATP by actomyosin was Ca^{2+} dependent in each of the 4 preparations studied. Thus, specific ATPase activity was 36.4 ± 3.7 nmole Pi/mg/min when Ca^{2+} was replaced with 2 m*M* EGTA ($P < 0.001$).

As shown in Fig. 1, isoelectric focusing of actomyosin revealed prominent bands at pH 5.62, 5.56, 5.05, 4.67 and 4.58, and a triplet with overlapping staining at pH 4.87. In each of four preparations studied, a faint band was located at pH 5.59, about midway between the doublet at pH 5.62 to 5.56. The positions of these three bands between pH 5.62 and pH 5.56 were similar to the multiple bands of actin described in preparations from chicken gizzard smooth muscle, unfused cultures of striated muscle cells, and nonmuscular contractile systems (19–21). In this context, the more acidic member of the group (pH 5.56) comigrated with actin purified from either rat skeletal or canine cardiac muscle (unpublished observations). The possibility that the existence of all three polymorphic forms of actin is a common feature of smooth muscle merits further study.

When ATP and Ca^{2+} were added to the reaction mixture and subjected to isoelectric focusing after incubation at 25° for 10 min

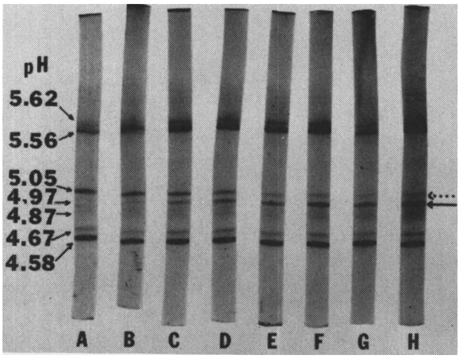

FIG. 1. Isoelectric focusing of bovine aortic actomyosin incubated in the presence of ATP (1 m*M*) and different concentrations of Ca^{2+}. The pH at which the major polypeptides focused is shown at left. A CaEGTA/EGTA buffer system was used as described in METHODS. In A, all of the Ca^{2+} was chelated with EGTA. The concentrations (*M*) of Ca^{2+} were 10^{-7} (B), 2×10^{-7} (C), 4×10^{-7} (D), 7×10^{-7} (E), 10^{-6} (F), 5×10^{-6} (G), and 9×10^{-5} (H). The parent band at pH 5.05 (dotted arrow) progressively decreased in intensity and a new band appeared at pH 4.97 (solid arrow) as Ca^{2+} was increased.

(Fig. 1), no significant change occurred in the bands at pH 5.62, 5.56, 4.87, 4.67 and 4.58. In sharp contrast, the band appearing at pH 5.05 markedly decreased in intensity and a new, more acidic, band appeared at pH 4.97. The extent to which this change occurred increased progressively as the concentration of Ca^{2+} in the reaction mixture increased (Fig. 1 and 4).

To test the hypothesis that the appearance of the new band at pH 4.97 was ascribable to phosphorylation, experiments were performed in the presence of ATP $\gamma^{32}P$ as described in METHODS. The results showed that significant radioactivity was present only in the new band at pH 4.97 (Fig. 2). No incorporation of ^{32}P occurred when Ca^{2+} was chelated with EGTA. These observations suggest that the parent band at pH 5.05 was phosphorylated in the presence of Ca^{2+} and ATP so that it was rendered more acidic and in this form focused at pH 4.97.

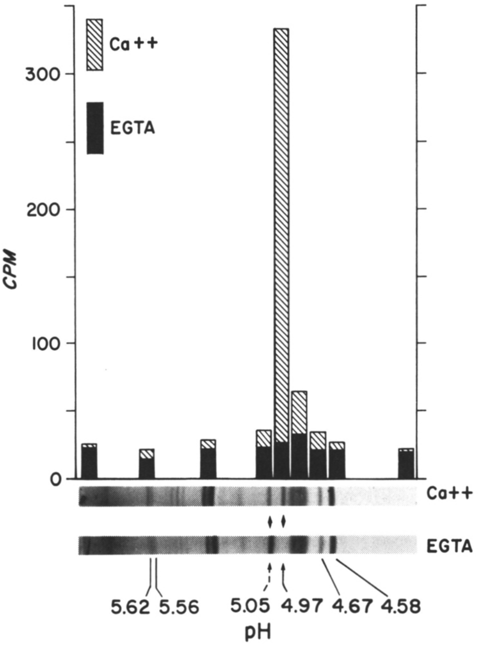

FIG. 2. Incorporation of ^{32}P from ATPγ^{32} (cpm, ordinate) is shown for different regions of isoelectric gels (shown at bottom) prepared from a reaction mixture containing 1 mg actomyosin/ml in the presence of 5×10^{-5} *M* Ca^{2+} (striped bars) the presence of 1 m*M* EGTA (solid bars). The sample applied to each gel containing 100 μg of protein. Note that a new band which incorporates ^{32}P appears at pH 4.97 in the presence of Ca^{2+}. Similar results were obtained with 3 different preparations.

SDS electrophoresis of the same reaction mixture containing ATP$\gamma^{32}P$ showed that incorporation of radioactivity was essentially limited to the band corresponding to the 15,000 myosin light chain (Fig. 3). It is tempting to suggest that that the parent band at pH 5.05 corresponds to the unphosphorylated 15,000 dalton light chain whereas the new band corresponds to the phosphorylated chain. However, the possibility that the phosphorylated and nonphosphorylated polypeptides represent moieties which co-migrate with the light chains cannot be excluded.

Our estimates of the molecular weights for the myosin light chains in aortic actomyosin (Fig. 3) are in accord with values reported for preparations from chicken gizzard (5, 8, 9) or platelets (10) and may reflect the heterogenity of myosin light chains isolated from various sources (22).

Two additional observations made with SDS electrophoresis are noteworthy. First, the SDS band pattern of actomyosin was not altered in the presence of ATP and Ca^{2+} (Fig.

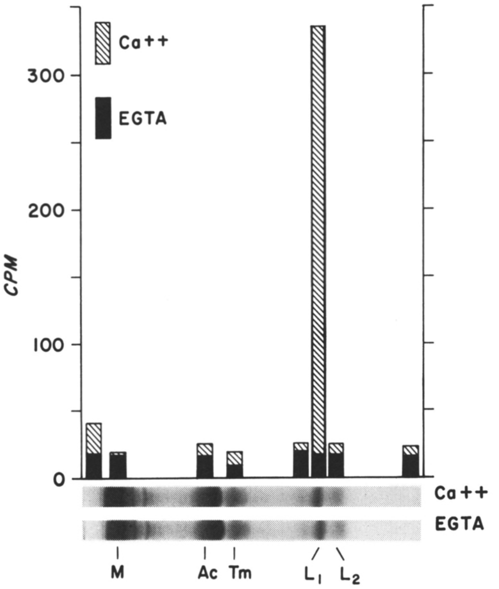

FIG. 3. Incorporation of ^{32}P from ATP$\gamma^{32}P$ (cpm, ordinate) is shown for different regions of SDS gels (shown at bottom) prepared from the same reaction mixtures used in Fig. 2. Striped bars show incorporation of ^{32}P in the presence of 10^{-5} *M* Ca^{2+} whereas solid bars show incorporation in the presence of 1 m*M* EGTA. Major bands and corresponding molecular weights (Daltons) are *M* (myosin 200,000), Ac (actin 45,000), Tm (tropomyosin in 37,000), L_1 (first light chain 15,000) and L_2 (second light chain 13,000).

3). This contrasts with findings obtained during isoelectric focusing (Fig. 2), and underscores the value of the focusing technique in distinguishing between phosphorylated and nonphosphorylated polypeptides. Second, no incorporation of ^{32}P into the myosin light chains (L_1 and L_2) occurred in the presence of EGTA (Fig. 3). This confirms findings obtained during isoelectric focusing showing that phosphorylation was Ca^{2+}-dependent (Fig. 2).

The Ca^{2+} requirement for phosphorylation of the parent band at pH 5.05 and its consequent movement to pH 4.97 is shown in Fig. 4 (solid curve). Densitometric measurements of coomassie-stained isoelectrically focused gels showed that 50% of total area under the parent band (pH 5.05) had moved to the location of the phosphorylated band at pH 4.97 at a Ca^{2+} concentration of 3×10^{-7} *M*. Similarly, 50% of the maximal activation of the Mg^{2+} stimulated actomyosin ATPase occurred at the same concentration of Ca^{2+} (Fig. 4, dashed curve). Phosphorylation and activation of ATPase was parallel over the entire range of Ca^{2+} concentrations tested.

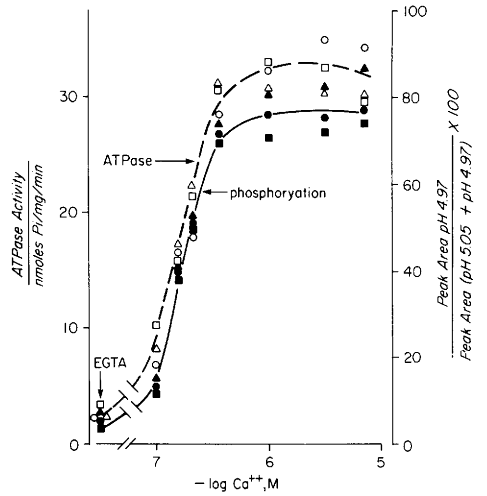

FIG. 4. Relationships are shown between the concentration of Ca^{2+} in the reaction mixture (abscissa) and specific activity of Mg^{++} stimulated actomyosin ATPase (left ordinate), and between Ca^{2+} and phosphorylated peptide detected during isoelectric focusing (right ordinate). Results are given for 3 different preparations identified by different symbols. The extent of phosphorylation is expressed as the ratio of the peak area of the phosphorylated polypeptide (pH 4.97) to the sum of the parent nonphosphorylated band (pH 5.05) plus the phosphorylated band (pH 4.97).

In conclusion, our findings show that phosphorylated and nonphosphorylated polypeptides in bovine aortic actomyosin can be easily distinguished by IEF on polyacrylamide gels. Although some degree of separation can be also achieved during electrophoresis on alkaline urea gels (12) IEF has a marked advantage in permitting determination of isoelectric points before and after phosphorylation. Furthermore, the separation of phosphorylated and nonphosphorylated polypeptides by IEF is sufficiently large so that an assessment can be made of the percent conversion of one form to the other.

Summary. Studies of bovine aortic actomyosin employing isoelectric focusing and sodium dodecyl sulfate electrophoresis show Ca^{2+} dependent phosphorylation of 15,000 dalton polypeptides. These polypeptides probably correspond to the myosin light chains. The Ca^{2+} requirement for phosphorylation parallels the Ca^{2+} requirement for activation of Mg^{2+} stimulated actomyosin ATPase. These findings suggest that the Ca^{2+} regulatory mechanism for actin-myosin interactions in mammalian vascular smooth muscle may be partly mediated by phosphorylation of myosin light chains.

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