Skinned cells of chicken gizzard were used to study the effect of a smooth muscle phosphatase (SMP-IV) on activation and relaxation of tension. SMP-IV has previously been shown to dephosphorylate light chains on myosin. When this phosphatase was added to submaximally Ca²⁺-activated skinned cells, tension increased while phosphorylation of myosin light chains decreased. In contrast, when the myosin phosphatase was added to cell bundles activated in the absence of Ca²⁺ by a Ca²⁺-insensitive myosin light chain kinase, tension and phosphorylation of the myosin light chains both decreased. These data suggest that Ca²⁺ inhibits the deactivation of tension even when myosin light chains are dephosphorylated to a low level. Further, comparison of Ca²⁺-activated cells caused to relax in CTP, in the presence or absence of Ca²⁺, shows that cells in the presence of Ca²⁺ do not relax completely, whereas in the absence of Ca²⁺ cells completely relax. Solutions containing Ca²⁺ and CTP, however, are incapable of generating tension from the resting state. Endogenous myosin light chain kinase is not active in solutions containing CTP and dephosphorylation of myosin light chains occurs in CTP solutions both in the presence and absence of Ca²⁺. These data imply that Ca²⁺ inhibits relaxation even though myosin light chains are dephosphorylated. These data are consistent with a model wherein an obligatory Ca²⁺-activated myosin light chain phosphorylation is followed by a second Ca²⁺ activation process for further tension development or maintenance.

Considerable evidence has accumulated showing that a correlation exists between myosin light chain phosphorylation and activation of tension in skinned smooth muscle fibers. The evidence is: 1) a close correlation exists between tension and myosin light chain phosphorylation or thiphosphorylation (1-3), 2) thiophosphorylation or phosphorylation of myosin light chains in the absence of Ca²⁺ is sufficient for maximal activation of tension (1, 2, 4), and 3) tension is affected in a parallel manner as myosin light chain phosphorylation by factors that affect the activity of myosin light chain kinase such as the activator calmodulin and inhibitors phenothiazines and catalytic subunit of cAMP-dependent protein kinase (3, 5, 6).

The myosin light chain kinase involved in the activation of smooth muscle fibers has been well characterized by several laboratories (7, 8). The characterization of the SMP involved in myosin light chain dephosphorylation is complicated because more than one SMP has been isolated (9-12). Two of these phosphatases, SMP-I and SMP-II, dephosphorylate isolated myosin light chains but not intact myosin and bind to neither myosin nor actin (13). Another phosphatase SMP-IV, shown to dephosphorylate myosin light chains on myosin as well as bind to myosin, has been purified and characterized (9, 13). This study shows that SMP-IV is capable of dephosphorylating myosin light chains in skinned chicken gizzard fibers and that the effect of myosin light chain phosphorylation on tension development is determined by the Ca²⁺ concentration.

**MATERIALS AND METHODS**

**Preparation of Skinned Cell Bundles**—Small bundles of skinned smooth muscle cells from the chicken gizzard were prepared by gentle homogenization with a glass tissue-homogenizing tube and Teflon pestle in a skinning solution as previously described (1, 2, 14). The ends of the pieces of cell bundles were inserted into small stainless steel clamps similar to the one used by Hellam and Podolsky (15). The cell bundles were then immersed in a relaxing solution containing 1% Triton X-100 (Sigma) for 20 min to solubilize the sarcolemma and sarcoplasmic reticulum. The cell bundles were then ready to be immersed into various test solutions for tension measurements.

**Test Solutions**—All test solutions contained 85 mM K⁺ + Na⁺, 2 mM MgATP²⁻, 1 mM Mg²⁺, 7 mM EGTA, 10⁻³-10⁻⁴ M free Ca²⁺, and propionate as the major anion. Imidazole propionate was used to maintain the pH at 7.00 ± 0.02 and to adjust the ionic strength to 0.15. Temperature was maintained at 23°C. Na⁺ was added as Na₄ATP. The concentrations of these species were determined by solving the ionic equilibrium equations using published binding constants for the various ionic species (17). When ATP-γ-S or CTP was used, they were direct substitutions for ATP in the test solutions. Relaxing solutions are solutions which contain no added Ca²⁺ (pCa = 9.0) and maximal contracting solutions are solutions that give maximal tension beyond which further increases in Ca²⁺ will not result in further tension (pCa = 3.8 in this study). A wash solution (pCa = 9, 7 mM EDTA, 85 mM K⁺ + Na⁺), and imidazole propionate to maintain pH of 7.00 and ionic strength of 0.15) was used as indicated in figures to quickly remove MgATP²⁻ from the fibers prior to transfer to ATP-γ-S or CTP-containing solutions. In some cases

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The column was washed with Buffer A containing 1 M KCl to elute immediately at 19,000 rpm for 5 min. The precipitate was extracted with Buffer A. Following centrifugation, the supernatant which contained the myosin light chains was cut out and laid under the negative electrode of a flatbed isoelectric focusing gel apparatus (Bio-Rad model 1415) and the light chains focused (19). The gels were stained with Coomassie Blue and dehydrated and autoradiographed to confirm the location of the \( ^{32}P \)-labeled phosphorylated light chains. The relative proportions of nonphosphorylated and phosphorylated myosin light chains were determined by autoradiography of SDS slab gels (1, 2).

Preparation of SMP-IV—SMP-IV was prepared from fresh turkey gizzard muscles by hydrophobic chromatography on an \( \epsilon \)-aminooctyl-Sepharose column equilibrated with 20 mM Tris-HCl (pH 7.4), 0.1 mM dithiothreitol, 75 mg/l of phenylmethylsulfonyl fluoride, 10 mg of soybean trypsin inhibitor, L-1-tryosylamido-2-phenylethyl ketone, and 1 mM of benzylarginyl/methyl ester, and 1 mg/l of leupeptin and pepstatin. The extract was subjected to (NH₄)₂SO₄ fractionation and the 30–60% saturation fraction was chromatographed on Sephacryl S-300. The column fractions were assayed for phosphatase activity with \( ^{32}P \)-labeled turkey gizzard light chains as substrates. SMP-I and -IV which eluted as a single peak of activity were further purified from other proteins on DEAE-Sephacel chromatography. The column fractions were treated with 5-fold its volume of 95% ethanol at room temperature. The sample was centrifuged at 19,000 rpm for 5 min. The precipitate was extracted with Buffer A containing 1 M KCl. The supernatant which contained SMP-I came off the column at 0.9 M KCl. SMP-IV was precipitated at 0.5 mM KC1 gradient, (b) Buffer A containing 500 mM KCl, and (c) 500–2000 mM KC1 gradient. SMP-IV was eluted at 0.4 M KC1 whereas SMP-I came off the column at 0.9 M KC1. SMP-IV was precipitated by addition of solid ammonium sulfate to 60% saturation at 0 °C. The precipitate was collected by centrifugation and dissolved in 5 ml of Buffer A. The resulting solution was treated with 3-fold its volume of 95% ethanol at room temperature. The sample was centrifuged immediately at 19,000 rpm for 5 min. The precipitate was extracted with Buffer A following centrifugation, the supernatant which contains SMP-IV was dialyzed against Buffer A. SMP-IV was further purified by affinity chromatography on a myosin-Sepharose column. The column was washed with Buffer A containing 1 M KC1 to elute SMP-IV. The enzyme was stored in Buffer A containing 50% glycerol at -20 °C.

RESULTS

The protein phosphorylation model for smooth muscle contraction predicts that dephosphorylating myosin light chains should speed up muscle inactivation and relaxation of muscle contraction. To test this model, SMP-IV was added to a skinned chicken gizzard cell bundle preparation submaximally activated at pCa = 5.2. Fig. 1A shows that the addition of the myosin phosphatase did not result in a decrease in tension but rather increased tension. This effect is reversed by removal of the phosphatase. Maximal tension is developed when the cell bundles are incubated at pCa = 3.8. In contrast, when a skinned cell bundle was contracted in the absence of Ca²⁺ by Ca²⁺-insensitive myosin light chain kinase, the addition of SMP-IV relaxed the cell bundles (Fig. 1B).

Although the effect of SMP-IV on tension was opposite in these two cases (Fig. 1. A and B), SMP-IV decreased phosphorylation of myosin light chains in both cases (Table I). In the absence of Ca²⁺, dephosphorylation of the myosin light chains relaxed the cell bundles, while in the presence of Ca²⁺, tension increased.

To characterize the combined effect of endogenous phosphatase activity and Ca²⁺ on smooth muscle tension and myosin dephosphorylation, the following experiment was per-
Myosin Phosphatase: Effect on Gizzard Skinned Fibers

Fig. 2. Time course of relaxation and dephosphorylation following maximum activation in the presence of pCa = 3.8 and ATP. After maximal phosphorylation (pCa = 3.8 and ATP, ○), dephosphorylation was followed with time under the following conditions: pCa = 9, ATP, ○; pCa = 9, CTP, Δ; and pCa = 3.8, CTP, □. A curve is drawn through the triangles (---). Three contractions are superimposed and followed by relaxation in pCa = 9, ATP, ——; relaxation in pCa = 9, CTP, ——; and partial relaxation in pCa = 3.8, CTP, ——; followed by complete relaxation in pCa = 9, CTP, ——. CTP is not used as a substrate for myosin light chain kinase but does serve as a substrate for tension activation (see Fig. 3). Maximal phosphorylation was 0.22 mol of phosphate/mol of LCm. Maximum tension was 101 mg for relaxation in pCa = 9, ATP; 102 mg for relaxation in pCa = 9, CTP; 40.2 mg for relaxation in pCa = 3.8, CTP.

Fig. 3. Tension versus time record showing activation of tension in pCa = 9 CTP following full thiophosphorylation in pCa = 3.8, ATPγS. All pCa solutions contain MgATP except where substitutions are indicated. Horizontal calibration bar, 5 min; vertical calibration bar, 26.9 mg. The wash solution is described under "Materials and Methods."

Fig. 4. Tension versus time record of a fiber bundle partially relaxed in pCa = 3.8, CTP. After a quick release in tension (R), the fiber bundle was able to redevelop active tension, showing that the tension is not due to noncycling cross-bridges. Horizontal calibration bar, 5 min; vertical calibration bar, 37.9 mg (at end of trace after 2 initial decreases in amplifier gain during the contraction in pCa = 3.8, ATP).

This Ca2+-dependent tension level observed in the presence of CTP is 50%–60% of the maximum tension that the cell bundle can support in the presence of CTP when the myosin light chains have been exposed to experimental conditions (1, 2) which maximally thiophosphorylate them (Fig. 3). Fig. 3 also shows that ATP, CTP, and ATPγS serve as substrates for actomyosin. However, they differ in the amount of tension they can support. The Ca2+-dependent tension observed at high Ca2+ in CTP (Fig. 2) is active since tension returned to its previous level following a quick release of a similar fiber to a shorter length (Fig. 4).

It would appear that phosphorylation of the myosin is a prerequisite for tension development and for the Ca2+ activation of actomyosin because incubation of the cell bundles in high Ca2+ (pCa = 3.8) in the presence of CTP is incapable of activating tension development from the relaxed state (Fig. 5).
phosphorylation. Except where indicated, all pCa solutions contain low (pCa 5) CTP or ATP. Fibers cannot be activated in CTP without prior phosphorylation. Except where indicated, all pCa solutions contain ATP. The wash solution is described under "Materials and Methods." Horizontal calibration bar, 5 min; vertical calibration bar, 12.8 mg.

![Graph showing tension vs. time for fibers treated with low (pCa = 9) and high (pCa = 3.8) calcium in the presence of CTP or ATP. Fibers cannot be activated in CTP without prior phosphorylation.](image)

**Fig. 5.** Tension versus time record of fibers treated with low (pCa = 9) and high (pCa = 3.8) calcium in the presence of CTP or ATP. Fibers cannot be activated in CTP without prior phosphorylation. Except where indicated, all pCa solutions contain ATP. The wash solution is described under "Materials and Methods." Horizontal calibration bar, 5 min; vertical calibration bar, 12.8 mg.

The data presented here show that dephosphorylation of the myosin light chains in the presence and absence of Ca^{2+} results in relaxation only in the absence of Ca^{2+} while in the presence of Ca^{2+} tension is maintained. These data suggest that there may be two components responsible for tension development: prior phosphorylation of myosin and Ca^{2+}-activated actomyosin.

**DISCUSSION**

The work presented in this paper helps to explain the relationship between the results from intact smooth muscle and skinned smooth muscle preparations. Also, it lends support to the models for smooth muscle contraction which postulate an additional Ca^{2+}-dependent mechanism being responsible for the activation of smooth muscle (23-28).

A model is presented (Fig. 6) and is used to explain the results obtained in this study. After phosphorylation of myosin, activation of the myosin filament occurs which makes it capable of interacting with actin to produce tension in the absence of Ca^{2+} (I, Fig. 6). This idea was initially supported in skinned cell preparations by our results showing that thiophosphorylated gizzard fibers were maximally activated in the absence of Ca^{2+} with MgATP^3- and that this activation was irreversible due to the inability of the endogenous phosphatase to remove the thiophosphate from the myosin light chains (1, 2). This idea also is supported by our previous observations that the Ca^{2+}-insensitive myosin light chain kinase is able to phosphorylate myosin light chains and activate maximum tension in the absence of Ca^{2+} (4). It is further supported by our observation in this study that fibers activated in the absence of Ca^{2+} by myosin phosphorylation can be relaxed by dephosphorylation using SMP-IV (Fig. 1B).

Evidence for another Ca^{2+}-activated state of the actomyosin filaments following dephosphorylation in the presence of Ca^{2+} (II) is presented in this study (Fig. 1A). This Ca^{2+}-activated state results from cycling myosin through phosphorylation in the presence of Ca^{2+} and is capable of producing tension. Thus, activation can exist in equilibrium between states I and II, both capable of producing tension. When a skinned cell bundle is immersed in high Ca^{2+} and the kinase is inactive (presence of CTP), no tension occurs (Fig. 5) unless myosin light chains are first phosphorylated (Fig. 2) or thio phosphorylated (Fig. 3). After the skinned cells have been activated in high Ca^{2+} and ATP, they can maintain a large percentage of the maximum tension in CTP (myosin light chain kinase not active) and high Ca^{2+} even in the absence of myosin light chain phosphorylation (Fig. 2). This suggests that the actomyosin can be locked into a Ca^{2+}-activated state (II) following myosin light chain phosphorylation and dephosphorylation which can generate tension with CTP. In the absence of Ca^{2+} both active states are inactivated, one (II) by removal of Ca^{2+} from the filaments (Fig. 2) and the other (I) by dephosphorylating the myosin light chains (Fig. 1B).

Previous work (1, 2, 21) has shown that under conditions of maximal thiophosphorylation (90%), Ca^{2+} does not activate the fibers further. This is not inconsistent with our model. Perhaps the hypothesized other Ca^{2+} activation site is on the thin filament. A cooperative interaction between myosin activation and thin filament Ca^{2+} activation could exist such that fully phosphorylated myosin does not require an additional activation of the thin filament to yield maximal actomyosin interactions (I). Once the actomyosin interactions have been fully activated by phosphorylation, you would not expect another activating system (a second Ca^{2+} activating step) to activate further. On the other hand, a submaximally activated system could be further activated by another Ca^{2+} activating system (II).

Previous work has shown that there is a close relationship between myosin light chain phosphorylation and tension in skinned smooth muscle cells but that the stoichiometry for maximal tension was low, approximately 0.2 (1, 2, 4, 6) to 0.5 (29) mol of phosphate/mol of myosin light chains for near maximum tension development. The low stoichiometry can easily be explained by this model (Fig. 6) which hypothesizes that there are two states (I, phosphorylated myosin; II, Ca^{2+}-activated actomyosin) in equilibrium. This would also explain why Ca^{2+} or Sr^{2+} activation of the cells occurs over a very small concentration range (1, 30). Such a system would show considerable positive cooperativity. Such a model suggests that for a given tension there could be many different stoichiometries of myosin light chain phosphorylation depending upon the Ca^{2+} concentration and the relative activity levels of the myosin light chain kinase and phosphatase.

This may help to explain why investigators working with intact smooth muscle preparations find differences in the relationship between the stoichiometry of myosin light chain phosphorylation and tension development in the muscle depending upon time and muscle type (24, 25, 28). Within a muscle there could be a high stoichiometry relationship between myosin light chain phosphorylation and tension early during contraction and as actomyosin filaments are converted...
to a Ca\textsuperscript{2+}-activated dephosphorylated form (II), the stoichiometry of myosin light chain phosphorylation to tension decreases.

One can only speculate as to the nature of the Ca\textsuperscript{2+}-activating mechanism occurring subsequent to the phosphorylation step. Possibly it involves leptomycin (26) or another protein (27) associated with the actin filament. In such a case, myosin interacting with the thin filament could serve as a catalyst to activate the filament. Alternatively, the Ca\textsuperscript{2+}-activating site could be associated with myosin (31). Under certain circumstances, smooth muscle myosin has been shown to bind 2 mol of Ca\textsuperscript{2+} per mol of myosin and be associated with the activation of actomyosin ATPase (31). Conceivably, a few phosphorylated myosin molecules on the thick filament could cooperatively interact with other myosin molecules allowing them to bind Ca\textsuperscript{2+} and to further activate the system. Our data cannot distinguish between any of these proposed models, but allows us to make some general statements about certain features of the model which we have already discussed.

The model presented here is very similar to the model which has been postulated by Murphy and co-workers (32, 33). Their model postulates that there are two states of myosin, rapidly cycling phosphorylated cross-bridges and slowly cycling Ca\textsuperscript{2+}-activated cross-bridges. Their model is based upon observations that there is a strong correlation between maximum velocity of shortening and myosin light chain phosphorylation, and a negative correlation between tension and myosin light chain phosphorylation following activation of the muscle by different agonists (34). More recent data from Murphy’s laboratory (29) using skinned smooth muscle cells showed a higher degree of tension for the same level of Ca\textsuperscript{2+} and myosin light chain phosphorylation following maximal Ca\textsuperscript{2+} activation than before maximal activation. The authors cite these data as evidence for support of their model. These results are consistent with our data showing that a high Ca\textsuperscript{2+} concentration and CTP can support tension only after maximum activation by Ca\textsuperscript{2+} and ATP. Data from Stull’s laboratory show in vivo that the initial phosphorylation levels during smooth muscle activation correlate well with later steady state isometric tension measurements after substantial dephosphorylation of myosin light chains has occurred (35). These data are cited as consistent with the hypothesis that obligatory phosphorylation of myosin light chains is required for smooth muscle activation. Our data strongly support the basic features of these models although no data are presented here regarding relative cycling rates of the different states.

By manipulating Ca\textsuperscript{2+} concentrations, myosin light chain kinase, and phosphatase activity in skinned gizzard cells using Ca\textsuperscript{2+}-insensitive myosin light chain kinase, SMP-IV, CTP, and ATP\textsubscript{S}, we show evidence suggesting the co-existence of two activated states, phosphorylated myosin and Ca\textsuperscript{2+}-activated actomyosin following myosin phosphorylation and dephosphorylation. The major evidence is: 1) submaximally Ca\textsuperscript{2+}-activated tension can be increased when the myosin light chains are dephosphorylated by addition of SMP-IV, 2) maximally Ca\textsuperscript{2+}-activated skinned cell bundles remained contracted in high Ca\textsuperscript{2+} and CTP following dephosphorylation of the myosin light chains, 3) contracting cell bundles relax in the absence of Ca\textsuperscript{2+} regardless of the state of activation.

REFERENCES