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# Exogenous Calmodulin Increases $Ca^{2+}$ Sensitivity of Isometric Tension Activation and Myosin Phosphorylation in Skinned Smooth Muscle*

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**Abstract.** We have investigated the effect of exogenous calmodulin on chicken gizzard or rabbit ileum smooth muscle functionally skinned by mechanical grinding or exposure to Triton X-100 detergent. We found that a specific protein inhibitor, modulator binding protein, caused a loss of $Ca^{2+}$-activated tension which was restored by subsequent treatment with calmodulin. Calmodulin at 5 μM increased 10-fold the speed of development of isometric tension while it had no significant effect on the rate of relaxation or on maximum tension at high $Ca^{2+}$ concentrations. The $Ca^{2+}$ sensitivity of steady state tension and $LC_{20}$ phosphorylation were also increased by 5 μM calmodulin. These results are consistent with a calmodulin-regulated light chain kinase/phosphatase system being responsible for activation of tension in smooth muscle.

**Key words:** Calmodulin – Skinned smooth muscle – Myosin phosphorylation

## Introduction

Calmodulin, the 17,000 dalton acidic $Ca^{2+}$-binding protein first identified as the activator of phosphodiesterase, is now known to be involved in the calcium regulation of many enzyme systems (Cheung 1980b). Myosin light chain kinase, the enzyme implicated as the $Ca^{2+}$-sensitive control mechanism for smooth muscle contration, is composed of a high molecular weight catalytic subunit and calmodulin, whether isolated from smooth (Dabrowska et al. 1978) or skeletal muscle (Yagi et al. 1978).

Ebashi's group has suggested an alternative activation mechanism involving a $Ca^{2+}$-sensitive thin filament component called leiotonin C (Mikawa et al. 1978; Ebashi 1980). Previous work in our laboratory using functionally skinned smooth muscle fiber bundle preparations is consistent with a $Ca^{2+}$-sensitive myosin light chain kinase/phosphatase system regulating contraction (Cassidy et al. 1979, 1980; Hoar et al. 1979; Kerrick et al. 1980a, 1981a). In the course of investigations concerning the ability of phenothiazine drugs to inhibit both $Ca^{2+}$ activation of tension and myosin phosphorylation in skinned smooth muscle (Cassidy et al. 1980; Kerrick et al. 1980a, 1981a), we noticed that chicken gizzard, or rabbit ileum and pulmonary artery preparations, which had relaxed in the presence of high $Ca^{2+}$ and phenothiazine, did not regain tension, or regained it very slowly, when the drug was removed from the bathing medium. However, when calmodulin was added, $Ca^{2+}$-sensitive tension was regained. These results led us to question whether exogenous calmodulin could affect the $Ca^{2+}$ sensitivity of noninhibited skinned smooth muscle. This would be expected if a $Ca^{2+}$-calmodulin complex interaction with myosin light chain kinase is required for activation of tension. We have found that added calmodulin greatly affects $Ca^{2+}$ control of vertebrate skinned smooth muscle of a variety of types in a manner consistent with a $Ca^{2+}$-calmodulin-myosin light chain kinase complex being responsible for the $Ca^{2+}$ activation. Recently Sparrow et al. (1981) have reported that exogenous calmodulin strikingly increases the $Ca^{2+}$ sensitivity, rate of contraction, and maximum tension in detergent skinned guinea pig taenia coli. In general, their results are very similar to those reported here.

## Materials and Methods

*Solutions.* The EGTA-buffered $Ca^{2+}$ solutions used for tension and phosphorylation measurements were similar to those used by Kerrick and Krasner (1975) but modified for smooth muscle skinned fibers (Cassidy et al. 1979; Hoar et al. 1979). They contained $K^+$, 85 mM; EGTA, 7 mM; $Mg^{2+}$, 1 mM; $MgATP^{2-}$, 2 mM; $Ca^{2+}$, $10^{-8}$–$10^{-3}$ M; pH 7.0; ionic strength 0.15; imidazole 82–84 mM; major anion propionate. Relaxing solution contained $10^{-8}$ M $Ca^{2+}$.

*Skinned Smooth Muscle Preparations.* Tension measurements were performed on an isometric tension transducer similar to that used by Hellam and Podolsky (1969) and experimental protocol, data collection and analysis of tension data were as previously described (Kerrick and Krasner 1975; Best et al. 1977). Chicken gizzard functionally skinned cell bundles were made by mechanically grinding approximately (1–2 mm)$^3$

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pieces of muscle in relaxing solution at 0°C in a glass homogenizer with teflon pestle (Hoar et al. 1979). Rabbit ileum strips (approximately $2 \times 0.2 \times 0.1$ mm) were prepared by dissecting as described (Cassidy et al. 1979), and transferred directly into relaxing solution which contained 1% w/v Triton X-100 (TX-100) at room temperature (24°C). After 1 h, the strips were transferred to relaxing solution without TX-100 and mounted in the transducer.

*Myosin Phosphorylation.* Myosin $LC_{20}$ light chain phosphorylation in skinned chicken gizzard was performed by incubating skinned cell bundles in solutions identical to those used for tension measurements except that [$\gamma - {}^{32}$P]ATP was added so that the final specific activity was 0.1–0.2 Ci/mMole. Phosphorylation by the endogenous myosin light chain kinase was stopped by quick transferral of the samples to SDS sample buffer at 100°C as previously described (Cassidy et al. 1979; Hoar et al. 1979) and incubation at 100°C for 5 min. The solubilized proteins were separated by SDS gel electrophoresis and the stoichiometric phosphorylation levels of the $LC_{20}$ light chain were determined as previously described (Cassidy et al. 1979; Hoar et al. 1979). Maximum phosphorylation levels were checked with isoelectric focusing (Cassidy et al. 1980).

*Calmodulin and Other Proteins.* Calmodulin was purified from bovine or porcine brain by the method of Wang and Desai (1977) and chicken gizzard by the method of Lin et al. (1974) using a modification by Dean Malencik (manuscript in preparation). Troponin-C was purified from rabbit skeletal muscle by the method of Greaser and Gergely (1973) as modified by Kerrick et al. (1980b). Bovine serum albumin was Fraction V from Sigma Chemicals, St. Louis, MO, USA. Modulator binding protein was prepared from bovine brain by the method of Sharma et al. (1979).

## Results

*Inhibitors of Calmodulin-Dependent Processes.* Calcium activation of tension in skinned smooth muscle preparations is inhibited by substances which are known to interfere with calmodulin-dependent processes. We have previously shown that the phenothiazine drugs can relax skinned smooth muscle in the presence of maximally activating levels of $Ca^{2+}$ and that exogenous calmodulin can restore $Ca^{2+}$-activated tension in these preparations (Cassidy et al. 1980; Kerrick et al. 1980a). Another specific inhibitor, the bovine brain modulator binding protein (Cheung 1971) also inhibits the $Ca^{2+}$-activated tension in skinned rabbit ileum and the inhibition is relieved by transferring the preparation to exogenous calmodulin (Fig. 1). In both of the above cases the redevelopment of tension in calmodulin was faster than $Ca^{2+}$-activated tension before inhibition. For this reason we decided to investigate the effect of exogenous calmodulin on $Ca^{2+}$-activated tension in untreated fibers.

*Effect of Calmodulin on Speed of Tension Development.* Exogenous calmodulin greatly increases the speed of development of isometric tension in functionally skinned chicken gizzard and rabbit ileum preparations. Figure 2 shows that exogenous calmodulin abolished the 2 min lag before development of isometric tension in skinned rabbit ileum and caused a more rapid rate of tension development than in the

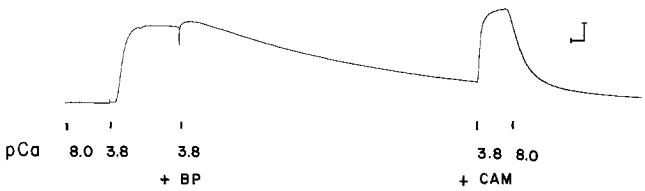


**Fig. 1.** Inhibition of $Ca^{2+}$-activated tension in skinned rabbit ileum by modulator binding protein (Sharma et al. 1979). 3.8 + BP = pCa 3.8 solution with modulator binding protein at 1 μM. 3.8 + CaM = pCa 3.8 solution with calmodulin at 5 μM. Calibration bars: vertical, 50 mg, horizontal, 5 min

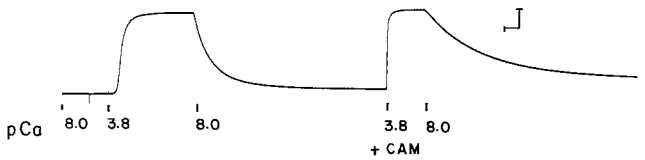


**Fig. 2.** Increase in speed of tension development in skinned rabbit ileum in the presence of exogenous calmodulin (CaM). A control tension cycle at left is followed by tension development in high $Ca^{2+}$ with added calmodulin. 3.8 + CaM = pCa 3.8 with calmodulin at 5 μM. Calibration bars: vertical, 50 mg; horizontal, 5 min

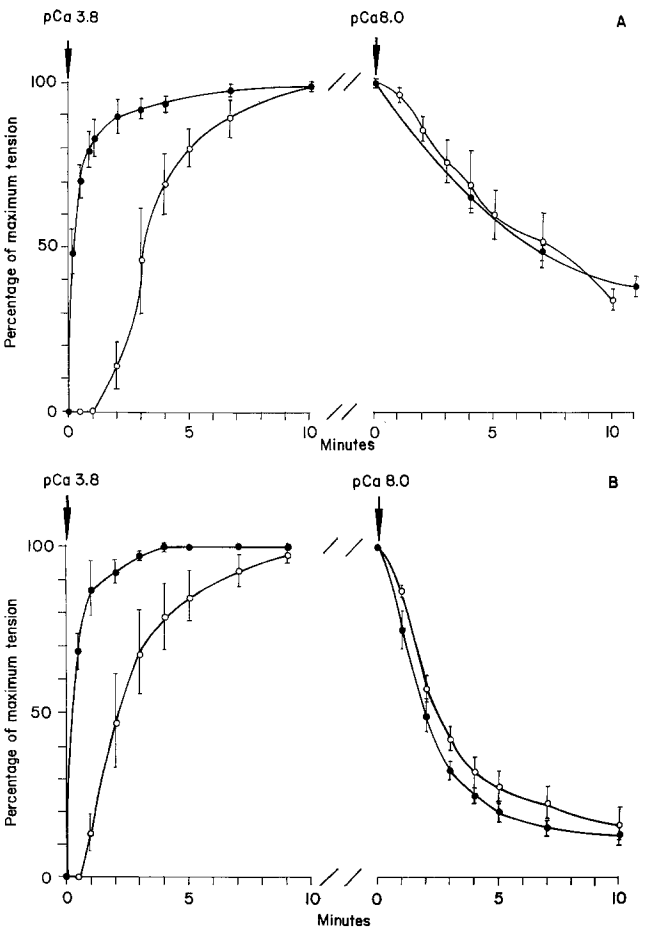


**Fig. 3A and B.** Time courses of tension development and relaxation in skinned smooth muscle with and without calmodulin. The data points represent the means of tensions developed by 5 preparations and the bars represent standard errors of the mean. *Arrows* at the top indicate the times at which the preparations were transferred to high $Ca^{2+}$ or low $Ca^{2+}$ solutions. (**A**) Rabbit ileum; (**B**) chicken gizzard; (○——○) no calmodulin; (●——●) calmodulin at 5 μM

control contraction. The maximum tensions attained in the absence and presence of calmodulin were the same. The mean tension values for 5 separate skinned preparations of rabbit ileum (Fig. 3A) and chicken gizzard (Fig. 3B) show that the presence of 5 μM calmodulin reduces the time necessary to reach 50% of maximum tension by about 10-fold. The presence of calmodulin had very little effect on the time course of relaxation when either smooth muscle preparation was relaxed in low $Ca^{2+}$ conditions (pCa = 8.0).

*Effect on Tension of Varying Calmodulin Concentration at Subthreshold $Ca^{2+}$ Concentration.* The effect of calmodulin on $Ca^{2+}$ sensitivity was measured directly by exposing the skinned smooth muscle preparations to a $Ca^{2+}$ solution which was below the threshold for tension development and then adding calmodulin. Figure 4 shows tension tracings of this type of experiment for both skinned gizzard and ileum. There was no or very little activition of tension of skinned gizzard at pCa = 5.6 or skinned ileum at pCa = 5.8. When the preparations are transferred into solutions containing 5 μM calmodulin, tension development was rapid and reached a steady state tension which was equivalent to the maximum tension reached under high $Ca^{2+}$ conditions. Tension was not activated by the addition of calmodulin in low $Ca^{2+}$ (pCa = 8.0) solution.

By varying the concentration of calmodulin in the pCa = 5.8 solution, a graded steady-state tension response was obtained (Fig. 5). Calmodulins prepared from three different sources (porcine brain, bovine brain, and chicken gizzard) activated tension at pCa 5.8 similarly in rabbit ileum (Fig. 5A) and chicken gizzard (Fig. 5B) and two other proteins [troponin subunit C and bovine serum albumin (BSA)] had no effect at concentrations up to 50 μM. If BSA was added at 1 μM to the calmodulin dilutions to protect against adsorption losses due to nonspecific protein binding sites, the activation of tension shifted to lower calmodulin concentrations, with the 50% tension midpoint shifting from pCaM = 6.6 to pCaM = 7.4 (Fig. 5C).

*Effect of Calmodulin on pCa-Tension Relationship and Myosin Phosphorylation.* We examined the effect of calmodulin on tension activation and phosphorylation of endogenous myosin $LC_{20}$ in skinned chicken gizzard muscle. Parallel experiments were performed on control preparations and preparations exposed to 0.1 and 5 μM calmodulin (Fig. 6A and B). The pCa at which 50% of maximum tension is reached was shifted from pCa 5 to pCa 5.7 in 0.1 μM calmodulin and to pCa 6.2 in 5 μM calmodulin. The pCa-phosphorylation curves were shifted similarly toward higher $Ca^{2+}$ sensitivity (lower $[Ca^{2+}]$) by calmodulin. As we have reported previously (Cassidy et al. 1979; Hoar et al. 1979) the level of phosphorylation of $LC_{20}$ in control fibers reaches approximately 20% of total $LC_{20}$ in preparations maximally activated in high $Ca^{2+}$ (pCa = 3.8) solution. Although maximum tension did not change with calmodulin treatment, maximum $LC_{20}$ phosphorylation increased 1.5- to 2-fold in the presence of 0.1 μM calmodulin and 3-fold in the presence of 5 μM calmodulin. The maximum shifts in pCa-tension and pCa-phosphorylation curves were obtained at 5 μM calmodulin.

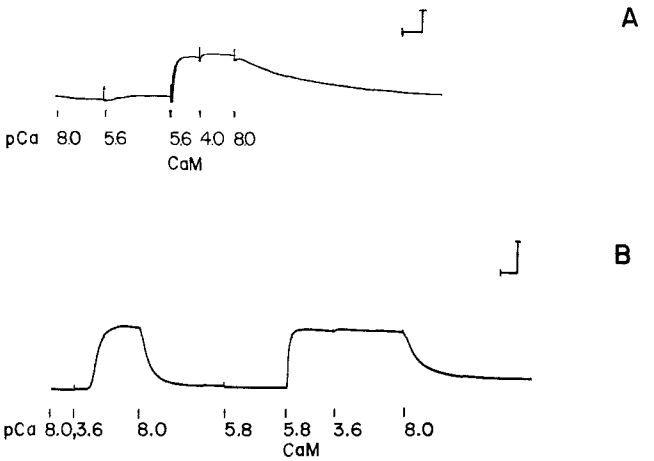


**Fig. 4A and B.** Tension records showing the increase in tension at a submaximally activating $Ca^{2+}$ concentration in skinned smooth muscle caused by added calmodulin. (**A**) Chicken gizzard. Calibration bars: vertical, 4 mg; horizontal, 5 min. (**B**) Rabbit ileum. Calibration bars: vertical, 50 mg; horizontal, 5 min. Calmodulin (CaM) at 5 μM

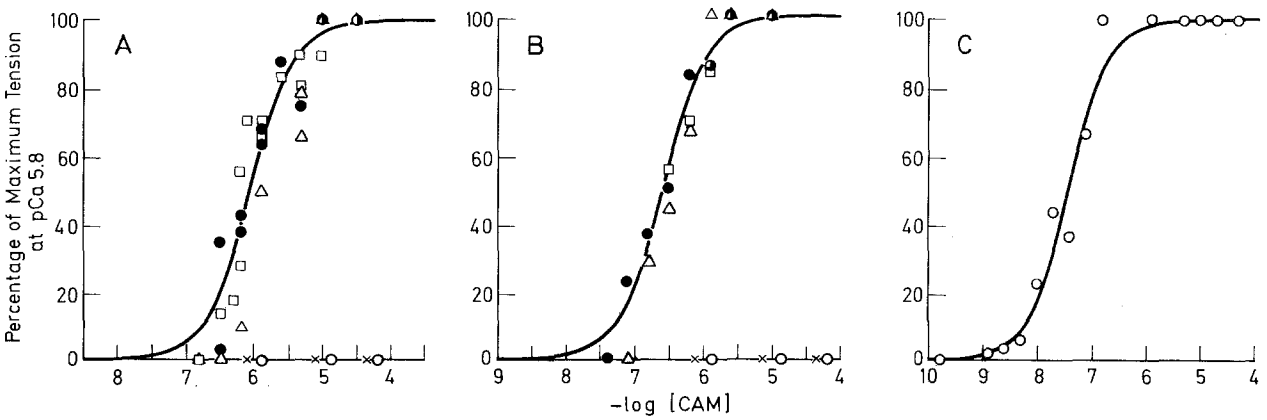


**Fig. 5A–C.** Effect of calmodulin (CAM) from different sources on steady state tensions at submaximally activating $Ca^{2+}$ concentration. (**A**) Rabbit ileum; (**B**) chicken gizzard. Experimental points (●), pork brain calmodulin; (□), beef brain calmodulin; (△) chicken gizzard calmodulin; (×), rabbit skeletal muscle TN-C; (○) bovine serum albumin at varying concentrations expressed as the negative log of molar concentration. (**C**) Chicken gizzard with 1 μM BSA added to all solutions; experimental points (○), pork brain calmodulin. Curve is computer generated fit to the Hill equation for the pork brain calmodulin points

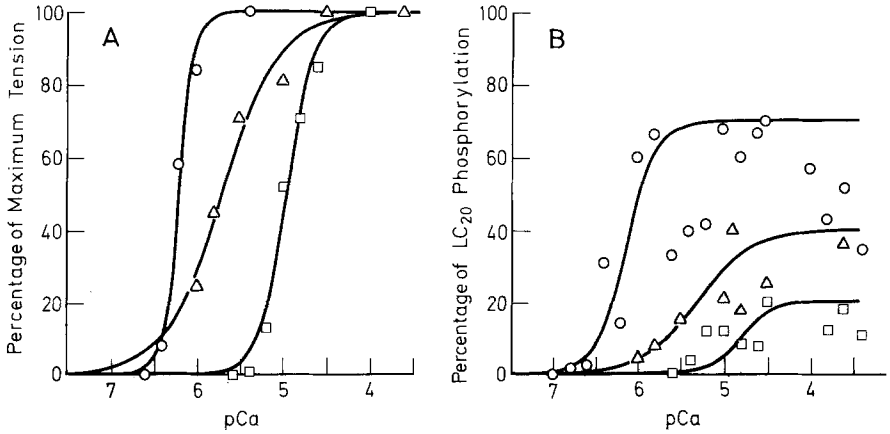


**Fig. 6A and B**
Effect of calmodulin on the pCa vs. tension relationship and myosin light chain phosphorylation in skinned chicken gizzard. (◇) Control; (△) calmodulin at 0.1 μM; (○) calmodulin at 5 μM. (**A**) pCa/tension curves. (**B**) pCa/myosin light chain phosphorylation curves. Phosphorylation is expressed as percentage of total $LC_{20}$ in the phosphorylated form

## Discussion

The existence of the calcium binding activator of phosphodiesterase called calmodulin was first discovered by its ability to reactivate an enzyme preparation partially depleted of calmodulin through ion exchange chromatography (Cheung 1971, 1980a). A number of $Ca^{2+}$-activated enzymes and ion pumps have been discovered and shown to be calmodulin dependent (Farrance and Vincenzi 1977; Cheung 1980b; Klee et al. 1980; Raess and Vincenzi 1980), by depletion of endogenous calmodulin and reactivation by exogenous calmodulin. We have found that addition of exogenous calmodulin to skinned smooth muscle preparations increases the $Ca^{2+}$ sensitivity in a manner very reminiscent of the reactivation of calmodulin depleted enzyme systems. Blumenthal and Stull (1980) have recently demonstrated that exogenous calmodulin can greatly increase the $Ca^{2+}$ sensitivity of purified myosin light chain kinase from rabbit skeletal muscle as would be expected for subsaturation amounts of calmodulin.

These results taken together may indicate that the skinning technique partially depletes the smooth muscle preparation of its endogenous calmodulin and that the $Ca^{2+}$ sensitivity of tension development observed in the control preparations is due to the small amount of calmodulin which remains after skinning. This model makes a number of assumptions. It assumes that the intracellular calmodulin total concentration is high in smooth muscle. Sharma et al. (1979) have estimated the concentration of calmodulin in bovine brain to be 5.0 μmol/kg. A similar calmodulin concentration may exist in gizzard in vivo, since calmodulin has been reported to represent 0.5–0.10% of the total tissue protein in smooth muscle (Adelstein and Eisenberg 1980); therefore this assumption seems valid.

If we assume that most of the total endogenous calmodulin in the cell is lost in the skinning process, we must further assume that enough myosin light chain kinase and the calmodulin remain bound in the skinned cell (perhaps at specific sites on the contractile proteins) and are responsible for the $Ca^{2+}$ sensitivity of tension development observed in skinned smooth muscle. The fact that two specific inhibitors of calmodulin mediated processes – phenothiazines and modulator binding protein – both inhibit this basal $Ca^{2+}$-activated tension is strong evidence that tightly bound endogenous calmodulin is responsible for activating the tension in skinned smooth muscle.

The skinned smooth muscle was prepared in a relaxing solution containing the $Ca^{2+}$ chelator EGTA at 7 mM. EGTA can fully dissociate calmodulin from phosphodiesterase (Cheung 1980b) or myosin kinase (Dabrowska et al. 1978; Adelstein and Klee 1980) catalytic subunits in solution. However, EGTA treatment was not sufficient to totally dissociate calmodulin from the particulate fractions of brain homogenate and even treatment with a nonionic detergent did not solubilize 100% of the brain calmodulin (Kakiuchi et al. 1978). Neither did EGTA treatment remove all of the ($Ca^{2+}$ + $Mg^{2+}$) ATPase activity in lysed human erythrocyte membranes (Kakiuchi et al. 1978).

The existence of a similar type of residual calmodulin in skinned smooth muscle could explain why smooth muscle preparations skinned in EGTA relaxing solution with or without 1% TX-100 detergent showed $Ca^{2+}$ sensitivity. It is possible that the remaining endogenous calmodulin slowly leaves the skinned smooth muscle cells and that this slow depletion is responsible for the gradual loss of $Ca^{2+}$ sensitivity which we have observed in gizzard fibers suspended in EGTA relaxing solution over a period of several hours.

The model assumes that the myosin light chain phosphorylation accomplished by the residual calmodulin-myosin-light chain kinase activity is sufficient to fully activate tension. This assumption is supported by the two interesting effects of exogenous calmodulin on the $Ca^{2+}$ sensitivities of tension activation and myosin light chain phosphorylation. First, both tension and light chain phosphorylation shift toward higher $Ca^{2+}$ sensitivities with increasing calmodulin concentrations. Second, much higher maximum levels of light chain phosphorylation attained with exogenous calmodulin are not associated with higher maximum tensions. These two results indicate that although light chain phosphorylation is increased by exogenous calmodulin and leads to an increase in submaximal tension, less than maximum light chain phosphorylation is required to maximally activate tension, and further increases in phosphorylation beyond a certain value (approximately 0.2 mol phosphate per mol $LC_{20}$) do not lead to increases in tension. So, although the speed of tension development of skinned smooth muscle fibers would be much slower than an in vivo contraction, eventually a high enough level of myosin phosphorylation would be reached to fully activate the skinned fiber. Exposure of such a skinned preparation to exogenous calmodulin would lead to a great increase in active myosin light chain kinase, presumably due to the binding of $Ca^{2+}$-calmodulin to the catalytic subunit of

myosin light chain kinase which remained bound in the fiber. This in turn would lead to increases in myosin light chain phosphorylation levels, speed of the tension development on exposure to high $Ca^{2+}$, and increased $Ca^{2+}$ sensitivity of steady state tension but would not lead to significantly higher maximum steady state tensions. Sparrow et al. (1981) have reported that exogenous calmodulin increases maximum tension at high $Ca^{2+}$ concentration by about 13%. We have occasionally observed small increases in maximal tension with exogenous calmodulin, but in general, we have not observed significant increases. In summary, a higher myosin light chain kinase activity at high $Ca^{2+}$-calmodulin concentration would increase the rate of light chain phosphorylation and hence of tension development, while on the other hand, the final tension would not be increased if tension production is already maximal at 20% phosphorylation of the light chains.

The same conclusion has been reached by us previously using a different experimental system based on the activation of skinned smooth muscle by ATPγS (Cassidy et al. 1979; Hoar et al. 1979; Kerrick et al. 1980a). Experiments using ATPγS have shown that about 80% of the $LC_{20}$ light chain in skinned gizzard of ileum preparations is available for phosphorylation by the small amount of endogenous myosin kinase (Cassidy et al. 1979). These experiments showed that skinned smooth muscle preparations were maximally activated when only about 20% of their $LC_{20}$ light chains were thiophosphorylated, and thiophosphorylation beyond this level did not lead to further increases in maximum steady state tension (Kerrick et al. 1980a). This may indicate either that the phosphorylatable myosin moves past a myosin light chain kinase possibly fixed to the thin filaments or that the myosin kinase is capable of limited diffusion within the fiber and slowly phosphorylates enough myosin to activate tension. So, by two independent methods – exposure to exogenous calmodulin or to ATPγS – we have shown that phosphorylation of $LC_{20}$ light chain to about 20% can fully activate tension in skinned smooth muscle.

This explanation of the results seems consistent with the great increase in the speed of tension development in calmodulin treated skinned smooth muscle preparations relative to controls. Smooth muscle in vivo and intact smooth muscle preparations in vitro contract within a time scale of seconds while our skinned preparation developed maximal tension in high $Ca^{2+}$ within a time scale of several minutes. Exogenous calmodulin treatment of skinned preparations returns the time scale to seconds, indicating that a concentration of calmodulin with the muscle preparation has been returned to an in vivo level. The dissociation constant of calmodulin for phosphodiesterase is in the range of $10^{-8}-10^{-9}$ M in solution (Cheung 1980b) and the concentration of calmodulin to achieve 50% activation of myosin light chain kinase is $10^{-9}$ M (Adelstein and Eisenberg 1980). The activating effect of calmodulin on our smooth muscle preparations is also produced at a very low calmodulin concentration between $10^{-8}$ and $10^{-7}$ M. The 10-fold discrepancy in these concentrations may be due to a slightly higher dissociation constant of calmodulin for the endogenous myosin light chain kinase in the fibers or simply a very slow equilibration of calmodulin concentrations in the fibers at very low solution calmodulin concentrations. At these low solution concentrations of calmodulin the intercellular concentration of calmodulin binding sites would be expected to approach that of the calmodulin concentrations in the bathing solutions and thus limit the rate of diffusion of calmodulin into the fibers. However, the concentration of calmodulin which half-activates myosin light chain kinase has also been reported by Yazawa et al. (1980) to be $3.16\times10^{-8}$ M which compares well with our value of $3.98\times10^{-8}$ M for half-maximal activation of tension in skinned smooth muscle fibers.

Hirata et al. (1980) have also found that when calmodulin was substituted for their proposed $Ca^{2+}$ binding activator of smooth muscle, leiotonin C, the superprecipitation of the myosin-leiotonin A-calmodulin complex was much more sensitive to $Sr^{2+}$ than the myosin-leiotonin A-leiotonin C complex or the original myosin-leiotonin complex. They reasoned that this suggested that the regulation of smooth muscle is carried out by leiotonin C and not calmodulin. However, their results as well as ours are consistent with a calmodulin regulated myosin light chain kinase/phosphatase system being responsible for the regulation of smooth muscle contraction.

The effects of exogenous calmodulin on skinned smooth muscle seem to be specific, since skinned mammalian striated and molluscan muscle fibers are unaffected by calmodulin (Kerrick et al. 1981a). The fact that a 17,000 dalton protein such as calmodulin and larger proteins such as the modulator binding protein, TN-I (Kerrick et al. 1976, 1981a), and cAMP-dependent protein kinase (Hoar and Kerrick 1980; Kerrick 1981b; Kerrick and Hoar 1981) can diffuse into skinned smooth muscle preparations and their effects easily studied indicates to us that this experimental approach may be used in the future to study the effects of the readdition of the myosin light chain kinase holoenzyme and catalytic subunits, myosin light chain phosphatase and other interesting proteins to skinned smooth muscle fibers. We believe that using this experimental approach with skinned smooth muscle preparations will lead to a clarification of our knowledge of the $Ca^{2+}$ activation mechanism(s) which control contraction and relaxation in smooth muscle.

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