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A Model for the Coregulation of Smooth Muscle Actomyosin by Caldesmon, Calponin, Tropomyosin and Myosin Light Chain Phosphorylation

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A model for the coregulation of smooth muscle actomyosin by caldesmon, calponin, tropomyosin, and the myosin regulatory light chain[1]

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The purpose of these studies was to evaluate the effects of the actin-binding proteins tropomyosin, caldesmon, and calponin on the activation of smooth muscle actomyosin by phosphorylation of the regulatory light chain of myosin (LC_{20}), and to interpret these findings in the context of a two-state kinetic model for the cross-bridge cycle. An in vitro motility assay was used to broadly classify each regulatory protein according to whether it modulates the apparent on-rate for cross bridges (f_{app}) or the apparent off-rate (g_{app}). In addition to measuring actin-filament velocity, a method was developed to measure relative changes in the force exerted on actin filaments under isometric conditions. Based primarily on the results of these motility studies, a qualitative model is proposed in which LC_{20} phosphorylation, tropomyosin, and caldesmon all regulate f_{app} and calponin regulates g_{app}. The model predicts that the sensitivity of activation by LC_{20} phosphorylation is determined by tropomyosin, caldesmon, and calponin, whereas unloaded shortening velocity is regulated primarily by calponin.

Key words: smooth muscle, caldesmon, calponin, tropomyosin, motility assay.

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Ces études ont eu pour but d'évaluer les effets des protéines de fixation de l'actine, tropomyosine, caldesmone et calponine, sur l'activation de l'actomyosine du muscle lisse par la phosphorylation de la LC_{20}, et d'interpréter ces résultats dans le contexte d'un modèle cinétique à deux états pour l'activité cyclique des ponts d'union. On a utilisé un test de motilité in vitro pour différencier les protéines régulatrices selon qu'elles modulent l'ouverture apparente (f_{app}) ou la fermeture apparente (g_{app}) de l'activité des ponts d'union. En plus de mesurer la vitesse des filaments d'actine, une méthode a été développée pour mesurer les variations relatives de la force exercée par les filaments d'actine dans des conditions isométriques. En se basant principalement sur les résultats de ces études de motilité, on propose un modèle qualitatif dans lequel la phosphorylation de la LC_{20}, la tropomyosine et la caldesmone régulent la f_{app}, alors que la calponine régule la g_{app}. Le modèle prédit que la sensibilité de l'activation de la phosphorylation de LC_{20} est déterminée par la tropomyosine, la caldesmone et la calponine, alors que la vitesse de raccourcissement sans charge est régulée principalement par la calponine.

Mots clés : muscle lisse, caldesmone, calponine, tropomyosine, test de motilité.

[Traduit par la Rédaction]

Introduction

In spite of the extensive pharmacologic literature on mammalian smooth muscles, the regulatory processes that govern the contractile proteins, actin and myosin, are not fully understood (Somlyo and Somlyo 1994). It is well established that covalent phosphorylation of the regulatory light-chain subunit (LC_{20}) of smooth muscle myosin is an important and perhaps obligatory activation mechanism. With very few exceptions, smooth muscle contraction has been found to be associated with some elevation in the level of LC_{20} phosphorylation (Kamm and Stull 1985). And yet, almost two decades after the original observation that LC_{20} phosphorylation activates the actin-activated myosin ATPase activity of smooth muscle and nonmuscle myosins (Adelstein and Conti 1975), no unique relationship between LC_{20} phosphorylation and any single parameter of smooth muscle contraction has been found that is consistent for all smooth muscles.

The goals of the studies to be described here were (*i*) to evaluate the general hypothesis that the actin-binding proteins tropomyosin, caldesmon, and calponin modulate the activation of contraction by LC_{20} phosphorylation; (*ii*) to broadly classify each regulatory protein according to whether it modulates the apparent on-rate for cross bridges (f_{app}) or the apparent off-rate (g_{app}); and (*iii*) to develop a qualitative model that describes the interaction of these regulatory proteins in the regulation of cross-bridge cycling under both isometric and unloaded conditions.

General considerations

The regulation of smooth muscle contraction is somewhat unique compared with the regulation of striated muscles because of the so-called latch state[3] that develops during sustained contractions. The latch state is a highly economical state of force maintenance that appears to be the result of a reduced rate of cross-bridge dissociation from actin, similar to the catch state described in certain invertebrate smooth muscles. Cardiac and skeletal muscles can also convert to a slow-cycling, high-economy contractile state. However, this involves the expression of specific myosin isoforms rather than dynamic

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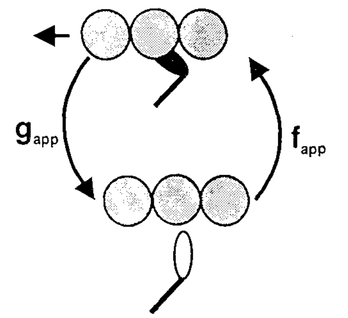
[3]The latch-bridge model, proposed by Dillon et al. (1981), suggested that dephosphorylation of an attached cross bridge would decrease the rate of cross-bridge dissociation from actin. There now is considerable evidence that the cross-bridge dissociation rate is regulated in smooth muscle independent of any changes in LC_{20} phosphorylation, suggesting an alternate mode of regulation. The term "latch state" will be used here more generally to refer to the slow-cycling cross-bridge state observed in smooth muscle, without regard to the underlying regulatory mechanism.



Relative Isometric Force = $f_{app}/(f_{app} + g_{app})$

Isometric ATPase = $(f_{app}g_{app})/(f_{app} + g_{app})$

Unloaded Velocity = $f(g_{app})$

Latch State = $f(g_{app})$?

FIG. 1. Two-state kinetic model of the cross-bridge cycle.

regulation as is seen in smooth muscle. In spite of these differences between smooth and striated muscles, there is no a priori reason to presuppose that the latch state in smooth muscle cannot be described by a simple two-state model of contraction in which both the on-rate and off-rate are regulated (Huxley 1957; Hai et al. 1988). As illustrated in Fig. 1, cross bridges can be broadly grouped into one of two general states. During the relaxed state, cross bridges are at equilibrium between a dissociated state and a weak binding state, where cross-bridge binding to actin is rapidly reversible and noncooperative (for review see Chalovich 1992). During contraction, cross bridges cycle through a strong-binding state, in which myosin cooperatively binds to actin with a 3000-fold higher affinity. Muscle shortening and (or) force development occur during the transition from the weak-binding to the strong-binding state.

In the Huxley (1957) model, the distribution of active cross bridges, under steady-state isometric conditions, is determined by the relative rates of cross-bridge attachment (f) and detachment (g). Because both the on-rate and the off-rate for cross bridges are now known to involve a number of discrete intermediate kinetic states, it is more appropriate to refer to an apparent on-rate (f_{app}) and an apparent off-rate (g_{app}) for the transition of cross bridges between the non-force-producing (i.e., dissociated and weak binding) states and the force-producing (i.e., strong binding) states (Brenner 1988). If the reverse rate constants ($-f$ and $-g$) are assumed to be negligible, then the level of isometric force is

$$\text{Normalized force} = \frac{f_{app}}{f_{app} + g_{app}}$$

An increase in f_{app} or a decrease in g_{app} of equal magnitude would each produce the same increase in force. However, the resulting contractile states would be different because the overall rate of cross-bridge cycling and the rate of ATP hydrolysis are proportional to $(f_{app} \times g_{app})/(f_{app} + g_{app})$ (Brenner 1988). Consequently, force development associated with an increase in f_{app} would be associated with a proportional increase in the rate of ATP hydrolysis, whereas force development associated with a decrease in g_{app} would be associated with a proportional decrease in the rate of ATP hydrolysis. For example, assuming that in the relaxed state $f_{app} \ll g_{app}$, an increase in f_{app} would increase both force and ATP hydrolysis. A subsequent decrease in g_{app} would cause a further increase in force, but this would be associated with a decrease in the rate of ATP hydrolysis back towards the resting level, or if both g_{app} and f_{app} decreased proportionately, force would be maintained by cross bridges cycling at a reduced rate. This is one possible model of how the latch state develops in smooth muscle. As a first step in the development of a model for the regulation of smooth muscle, several putative regulatory proteins were examined with regard to modulation of f_{app} and (or) g_{app}. This was accomplished through the use of an in vitro motility assay with modifications that provided for the measurement of unloaded filament velocity as well as relative changes in the force exerted on actin filaments under isometric conditions.

In vitro motility assay

The form of motility assay that we have used for this purpose is based on the movement of actin filaments over a surface of immobilized myosin similar to that described by Kron et al. (1991), with modifications to limit photochemical alterations of proteins. In all of the studies described here, smooth muscle myosin was thiophosphorylated to 2.0 mol PO_4/mol myosin and applied to a surface of nitrocellulose as monomeric myosin (300 mM KCl). Actin filaments were labeled with rhodamine–phalloidin and then bound to the myosin-coated surface under rigor conditions. After washing out unbound actin, an ATP-containing motility buffer was added to initiate filament motility. Tropomyosin, caldesmon, and calponin were included in the motility buffer. Velocity was measured at 30°C as described elsewhere, using a method that minimizes averaging errors for discontinuous filament motion (Haeberle et al. 1992).

In agreement with the Huxley (1957) model, unloaded filament velocity is limited by the rate of cross-bridge detachment (Warshaw et al. 1991) and is independent of the number of cycling cross bridges (Haeberle 1994). Consequently, if careful measures are taken to prevent filament loading in the motility assay, an increase in f_{app} should have no effect on velocity, but should cause a proportional increase in both force and the rate of ATP hydrolysis. An increase in g_{app}, in contrast, should cause an increase in both the rate of ATP hydrolysis and velocity, and a reduction in force.[4]

[4]The Huxley (1957) model contained two dissociation rate constants for nonisometric conditions: one for the dissociation of positively strained working cross bridges and a second for negatively strained cross bridges that had completed the work-producing portion of the cycle but which were still attached to actin. He argued that cross bridges become negatively strained prior to detachment during nonisometric contraction because other positively strained cross bridges continue to translocate the actin filament. He also assumed that negative strain increased the rate of cross-bridge dissociation. With this model, shortening velocity approached a limiting value when the total internal load due to negatively strained cross bridges equaled the total force generated by positively strained cross bridges. The rate of g_{app} is limited by the rate of ADP release under both isometric and isotonic conditions. Therefore, even though the values of g_{app} may be different under isometric and unloaded conditions, it is reasonable to assume that regulation of the rate of ADP release might change the dissociation rate under both conditions, and almost certainly would change both in the same direction. Since only directional changes in rate constants are being considered in the current model, it will not be necessary to differentiate between g_{app} for negatively and positively strained cross bridges.

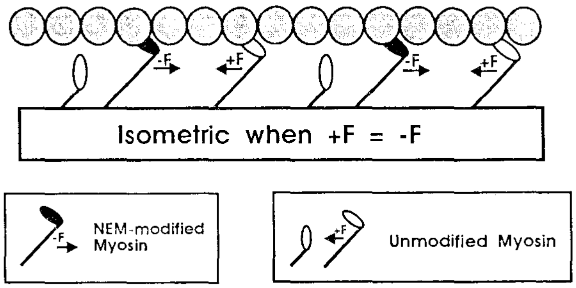


FIG. 2. Method for estimation of relative force ($Force_{NEM}$) using NEM-modified myosin. The minimum ratio of NEM-modified/unmodified myosin that prevented filament motion provided an index ($Force_{NEM}$) of the force generated by unmodified cross bridges. Changes in $Force_{NEM}$ with different experimental conditions provided a measure of relative changes in isometric force. $Force_{NEM}$ value (F_{NEM}) was independent of the myosin density on the cover slip (i.e., the concentration of NEM–myosin required to inhibit motility was linearly related to the concentration of unmodified myosin applied), but was sensitive to changes in force production by different myosin isoforms and to changes in activation by phosphorylation and by actin-linked regulatory proteins.

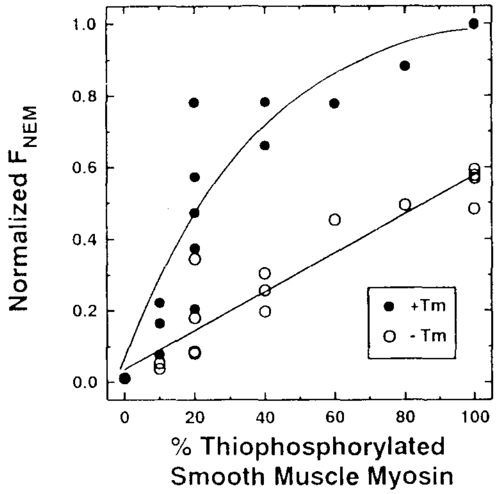


FIG. 3. Effect of smooth muscle tropomyosin on filament motility. Different levels of myosin thiophosphorylation were achieved by mixing appropriate amounts of dephosphorylated myosin and myosin that had been thiophosphorylated with myosin light chain kinase to 2.0 mol/mol. Values for $Force_{NEM}$ (F_{NEM}) were determined as described in Fig. 2 and by Haeberle (1994). The tropomyosin concentration was 1.8 μM.

As a consequence of these considerations, it should be possible to determine if a regulatory factor is predominately affecting f_{app} or g_{app} by measuring directional changes in isometric force, velocity, and actin-activated myosin ATPase activity. At this level of consideration, such measurements need only be accurate enough to resolve a significant positive or negative change.

Measurement of unloaded velocity and isometric force with the motility assay

The motility assay can provide relatively accurate measurements of changes in unloaded velocity and isometric force, but measurement of ATPase activity under the identical conditions that either force or velocity are measured is difficult because of the small amount of actin present. However, in almost all cases, measurements of force and velocity alone were sufficient to determine the rate constant that was regulated. Force development by single actin filaments has been measured using calibrated microneedles (Ishijima et al. 1991; VanBuren et al. 1993) or a laser light trap (Saito et al. 1994). These methods have the advantage that they provide absolute measures of force, but they are technically very demanding and not well suited to making the large number of measurements necessary to compare several different proteins over a range of concentrations and phosphorylation levels. Fortunately, for our purposes absolute force measurements were not necessary. With this in mind, a technically simpler method was developed to measure relative changes in the force exerted on actin filaments under isometric conditions (Fig. 2). *N*-Ethylmaleimide (NEM) modified skeletal muscle myosin was mixed with thiophosphorylated smooth muscle myosin to impose a mechanical load on the filaments. NEM modification of myosin produces a form of myosin that binds tightly to actin in the presence of Mg ATP, and impedes the motion of actin filaments with both skeletal and smooth muscle myosins (Warshaw et al. 1990). If a sufficient concentration of NEM–myosin was mixed with thiophosphorylated myosin, filament motion ceased. Using a method of successive approximations, it was possible to determine the minimum amount of NEM–myosin required to stop filament motion, and this provided an index of relative force ($Force_{NEM}$) (Haeberle 1994), where

$$Force_{NEM} = \frac{(\text{mol NEM-modified myosin})}{(\text{mol unmodified myosin})}$$

The $Force_{NEM}$ value for thiophosphorylated smooth muscle myosin was 3.4 times larger than for skeletal muscle myosin (Haeberle 1994) and compares favorably with the 3- to 4-fold force difference measured by VanBuren et al. (1993) using calibrated microneedles.[5]

While measurements of velocity are technically easier, they are not without pitfalls. Because of the steep velocity versus load relationship described by the force–velocity curve for muscle, it is important to minimize any stray mechanical loads, particularly when working at low myosin concentrations or at low levels of activation. The absence of mechanical loading by factors other than myosin itself can be verified by showing that velocity is independent of changes in the density of myosin on the cover slip. However, this does not provide an adequate test for mechanical loading by photochemically modified forms of myosin (e.g., cross bridges that bind to actin but do not cycle), because the load would change in exact proportion to the number of force-producing cross bridges. Ideally, velocity should also be independent of changes in f_{app}, and therefore, demonstration of velocity independence under conditions where f_{app} is regulated should provide a better test for mechanical loading. As will be discussed in later sections, our studies have shown that LC_{20} phosphorylation, caldesmon, and smooth muscle tropomyosin all regulate f_{app}, but not g_{app}, and have no effect on velocity when steps are taken to prevent photochemical modification. In contrast, all three altered velocity under more standard motility assay conditions in which velocity was nonetheless independent of myo-

[5]Further details of the method and control experiments demonstrating the validity of this approach for measuring relative force changes are presented elsewhere (Haeberle 1994).

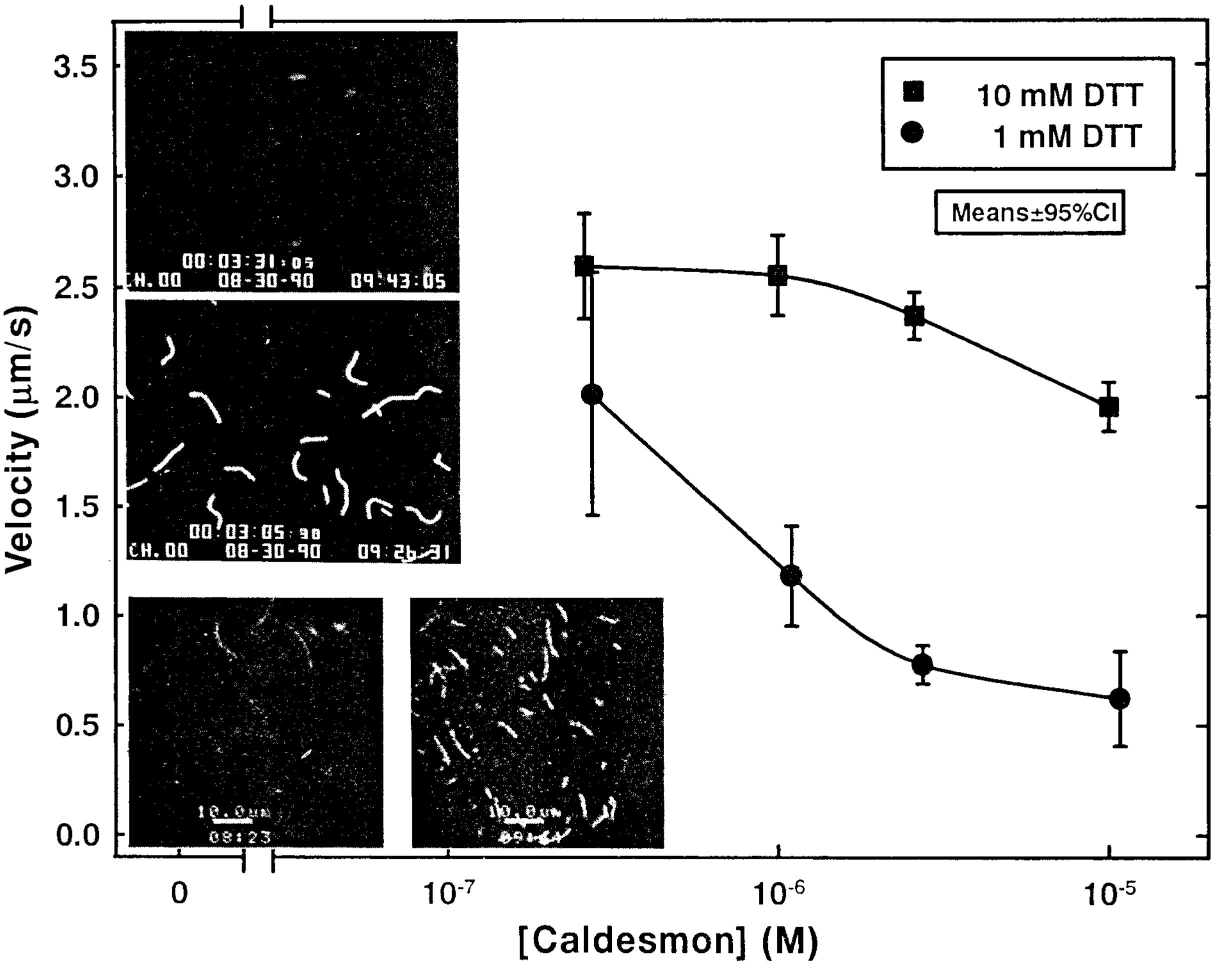


FIG. 4. Effect of intact caldesmon on filament binding and velocity. The inset panels show video images obtained by focusing the fluorescence microscope on the nitrocellulose-coated surface of the cover slip. In all the panels, the motility buffer contained 80 mM KCl, 1.6 μM tropomyosin and no added methylcellulose. Top panel, no added caldesmon; middle panel, 10 μM dephosphorylated chicken gizzard caldesmon; bottom left panel, dephosphorylated porcine aortic caldesmon; bottom right panel, porcine aortic caldesmon phosphorylated to 1.8 mol/mol with purified mitogen-activated protein kinase. Under these conditions filaments attached only in the presence of dephosphorylated caldesmon. The plotted data summarize the effects of caldesmon on filament velocity in the presence of either 1 or 10 mM DTT. The motility buffer was identical with that used for the binding studies. Data are presented as means with 95% confidence intervals. Adapted from Haeberle et al. (1992).

sin concentration. This clearly suggests that chemically modified myosin represents the major source of mechanical loading in the in vitro motility assay.

In our experience, the inclusion of a robust chemical system to scavenge both oxygen and free radicals while maintaining reducing conditions within the flow cell was essential to establish unloaded conditions. We found that either 1 mg BSA (bovine serum albumin)/mL + 100 mM DTT (dithiothreitol) or 20 mg BSA/mL + 10 mM DTT in degassed motility buffer containing an oxygen scavenger system markedly reduced photobleaching and mechanical loading was less than about 1% of the $Force_{NEM}$ produced by thiophosphorylated smooth muscle myosin (Haeberle et al. 1992).[6] Photobleaching was further reduced by the use of image enhancement techniques (i.e., frame averaging and background subtraction) that allowed for visualization of the filaments with reduced illumination.[7] Under these conditions, filament motility can readily be monitored for more than 30 min with continuous illumination.

Effect of LC_{20} phosphorylation on filament motility

At lower ionic strength, there is a complex relationship between velocity and the extent of LC_{20} phosphorylation that is due to mechanical loading by unphosphorylated, attached, weak-binding cross bridges (Warshaw et al. 1990). At higher ionic strength (100 mM), there was no significant (analysis of variance, $p < 0.05$) effect of LC_{20} phosphorylation on velocity from 1.0 to 0.05 mol PO_4/mol LC_{20} in the presence of tropomyosin if very careful attention was paid to the elimina-

[6]The addition of glucose, glucose oxidase, and catalase as an oxygen scavenger system (Kishino and Yanagida 1988; Kron et al. 1991) and 1 mM DTT actually enhanced the oxidation of some soluble proteins compared with 1 mM DTT alone, particularly if the solutions were not adequately degassed under vacuum (Haeberle et al. 1992). Presumably this was because the soluble proteins were present at 1 000 – 10 000 times the concentration of catalase, and would effectively compete with catalase for the peroxide generated by glucose oxidase.

[7]A 100-W mercury vapor lamp with a neutral density filter (5% transmittance) in the illumination path. In general, the level of illumination was reduced until the filaments could just barely be visualized using a standard epifluorescent microscope (Neofluar 100×/1.30 NA, Carl Zeiss Inc., Thornwood, N.Y.) equipped with a SIT camera (SIT-66, Dage-MTI Inc., Michigan City, Ind.).

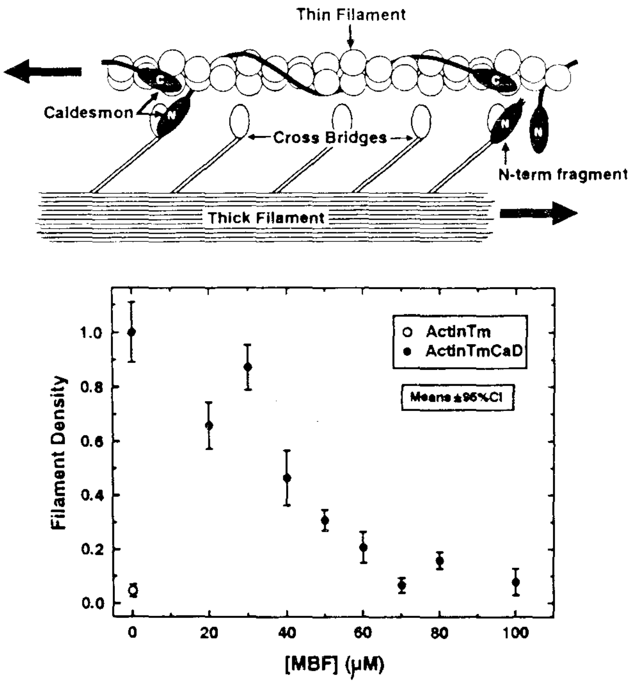


FIG. 5. Inhibition of caldesmon-dependent tethering by the N-terminal (N-term) myosin-binding fragment (MBF) of caldesmon (CaD). The binding of skeletal muscle actin filaments to thiophosphorylated smooth muscle myosin immobilized on a nitrocellulose-coated cover slip (i.e., an in vitro motility assay). The concentrations of tropomyosin and caldesmon were 1 and 0.4 μM, respectively. The N-terminal fragment of caldesmon was prepared by cleavage with 2-nitro-5-thiocyanobenzoic acid. Filament density was normalized to the filament density measured in the absence of caldesmon. Filament binding was quantified using a computer program to determine the number and length of bound actin filaments. These values were multiplied together to determine a value for total bound actin. All data were normalized to the control value determined in the presence of 0.4 μM intact caldesmon. ●, binding in the presence of 0.4 μM intact caldesmon and different concentrations of N-terminal fragment (MBF); ○, binding in the absence of both intact caldesmon and N-terminal fragment. The concentration of KCl in the motility buffer was 80 mM and that of tropomyosin was 1.6 μM. Methyl cellulose was not included. Values are presented as means with 95% confidence intervals. Adapted from Hemric and Haeberle (1993).

tion of all mechanical loading (unpublished results). When adequate measures were taken to limit chemical modification of contractile proteins, we determined that $\mathrm{Force_{NEM}}$ with fully thiophosphorylated myosin was 200-fold greater than $\mathrm{Force_{NEM}}$ with unphosphorylated myosin (unpublished results) and that $\mathrm{Force_{NEM}}$ is linearly related to the stoichiometry of LC_{20} phosphorylation (Fig. 3). This suggested that LC_{20} phosphorylation only affects f_{app}, in agreement with previous studies by Sellers (1985). These results are also consistent with the argument that unloaded velocity is governed primarily by g_{app}.

Effects of tropomyosin on filament motility

We have not been able to demonstrate any significant effect of tropomyosin on velocity in the motility assay using thiophosphorylated smooth muscle myosin, when adequate measures are taken to prevent mechanical loading. Although we earlier observed that tropomyosin increased filament velocity by approximately 50%, the measures we have since taken to eliminate photochemical modification have resulted in a progressive increase in filament velocity under all conditions and have eliminated the increase in velocity with the addition of tropomyosin. With our current protocol, we found that tropomyosin produced a modest increase in binding, and $\mathrm{Force_{NEM}}$ was increased by 67% (Fig. 3) with no effect on velocity (see control values in Figs. 6 and 7). This indicated that tropomyosin stimulates f_{app} but does not effect g_{app}. Presumably the effect on velocity observed in earlier studies reflected the fact that the filaments were not completely unloaded, and consequently, increasing f_{app} increased the force exerted on the filaments without changing the load, leading to increased velocity as predicted by the force-velocity curve.

The increased sensitivity of force activation by light-chain phosphorylation in the presence of tropomyosin suggests that smooth tropomyosin may function like skeletal tropomyosin to switch on the actin filament as described by Hill et al. (1981). These findings are consistent with previous studies by Somlyo et al. (1988) showing rigor-dependent force development in chemically permeabilized smooth muscle, as well as the report by Horiuchi and Chacko (1989) showing that tropomyosin enhances the cooperative switching on of actin by attached, high-affinity cross bridges. Vyas et al. (1992) have reported similar findings in chemically permeabilized smooth muscle fibers. However, they have interpreted their findings as evidence for cooperative activation mediated through the myosin filament.

Effects of caldesmon on filament motility

While there was initially some controversy concerning the effects of caldesmon on actin-activated ATPase activity, studies from Chalovich's laboratory (Chalovich 1992) have shown that the C-terminal end of caldesmon competitively inhibits myosin binding to actin and consequently inhibits actin-activated ATP hydrolysis. The apparent increase in acto-HMM (heavy meromyosin) binding that had previously been reported was shown to be due to the formation of an actin–caldesmon–HMM complex that tethers myosin to actin (Hemric and Chalovich 1990). We have verified both effects of caldesmon, using the motility assay.

Caldesmon tethers actin filaments to myosin and thereby facilitates motility at a higher ionic strength (100 mM) in much the same way that methylcellulose prevents diffusion of unbound filaments away from the myosin surface (Fig. 4, insets). The enhanced binding could be shown to be specifically due to tethering by using a molar excess of purified N-terminal fragment to competitively block caldesmon-dependent tethering (Fig. 5). This protocol demonstrated that the enhancement of binding was not due to an effect of caldesmon on the actomyosin interaction, because filament binding was decreased even though continued binding of both actin and myosin to caldesmon was assured by the presence of both intact caldesmon and the N-terminal fragment. Phosphorylation of the N-terminal fragment to 1.6 mol/mol by Ca^{2+}/calmodulin-dependent protein kinase II (Hemric et al. 1993) prevented the inhibition of tethering, and phosphorylation of intact caldesmon to 1.8 mol/mol by mitogen-activated protein kinase[8] prevented tethering (Fig. 4, inset).

[8]Phosphorylated bovine aortic caldesmon (Adam and Hathaway 1993) was provided by Dr. L.P. Adam (Indiana University School of Medicine).

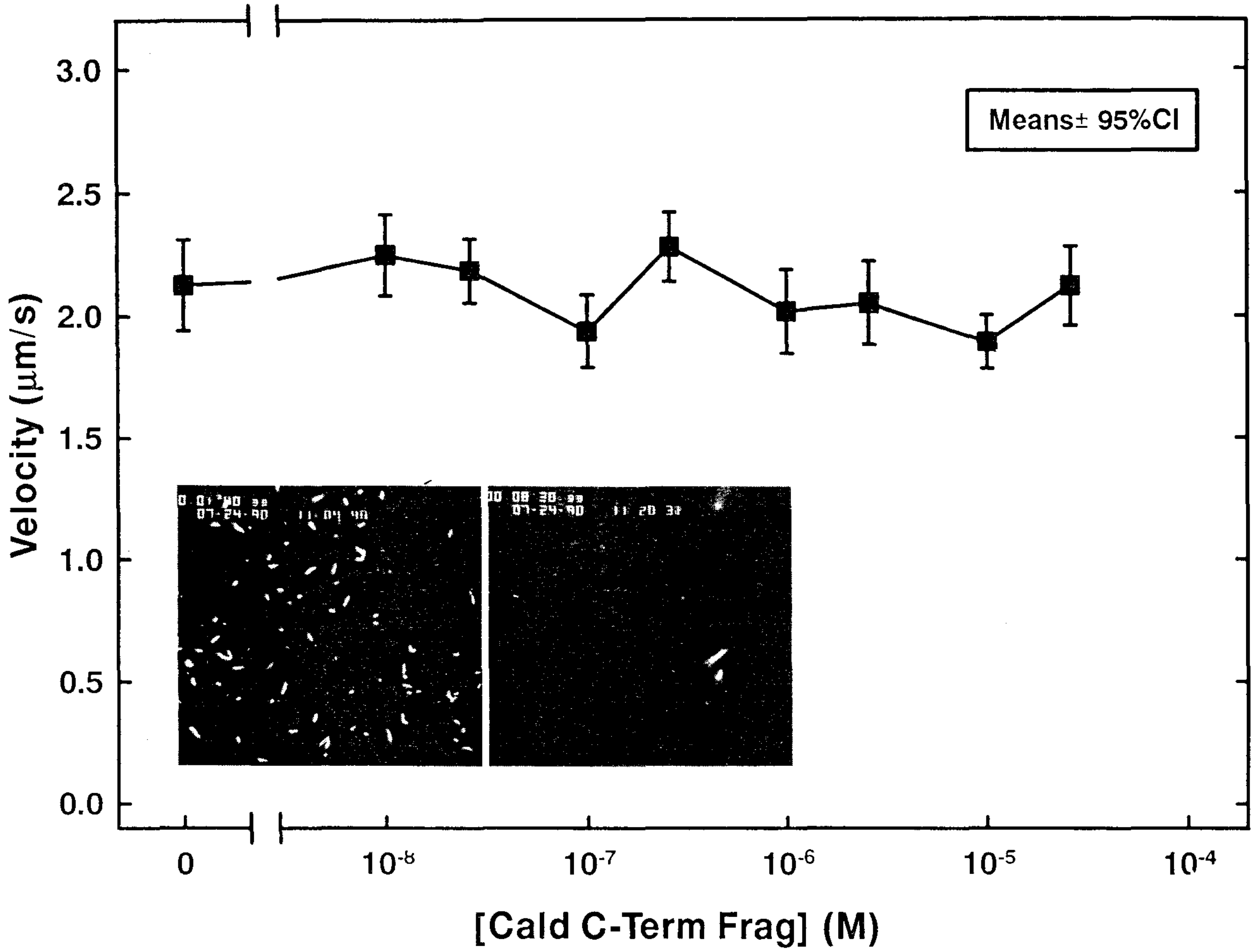


FIG. 6. Effect of C-terminal, actin-binding fragment of caldesmon (Cald C-Term Frag) on filament binding and velocity. The inset panels show the inhibition of filament binding to the myosin surface under low salt conditions. The concentration of KCl in the motility buffer was 25 mM and methylcellulose was not added. In the absence of fragment (left panel), filaments were bound tightly with little evidence of dissociation. The addition of 2.5 μM fragment completely reversed filament binding to the myosin surface (right panel). To determine the effects of the fragment on filament velocity (plotted data), 0.5% methylcellulose was included in the motility buffer to prevent diffusion of the filaments away from the myosin surface. Control studies have shown that methylcellulose alone has no effect on filament velocity. Adapted from Haeberle et al. (1992).

As shown in Fig. 6, a C-terminal fragment of caldesmon prevented binding of actin filaments to the myosin surface at low ionic strength (40 mM), but had no effect on filament velocity when 0.5% methylcellulose was added to the motility buffer to restrict diffusion of the filaments away from the surface.[9] Likewise, we found that intact caldesmon had no effect on filament velocity (Fig. 4). Since there was no binding or motility at 100 mM ionic strength in the absence of caldesmon, any filaments that bound and moved at this higher ionic strength in the presence of caldesmon must have been moving while tethered via caldesmon. At caldesmon concentrations >0.2 μM, filaments moved at 2.0–2.5 μm/s when 10 mM DTT was included in the motility buffer.[10] These results ruled out the possibility that the caldesmon tether might be a load-

[9]Because filament motion was very intermittent under these conditions, estimation of the true filament velocity was hampered by the limited sampling rate of the video camera and the limited spatial resolution of this imaging method (Work and Warshaw 1992). We have described elsewhere (Haeberle et al. 1992) methods to increase the sampling frequency and, therefore, to minimize velocity measurement errors under these conditions. Using these methods, velocity data were normally distributed ($p < 0.05$, χ^2 test) with a SD that was approximately 10% of the mean. In comparison, random sampling at a slower rate (i.e., 1–0.1 images/s) and calculation of velocity based on measured displacement between successive video images resulted in a lower mean velocity, a greater relative SD, and a velocity distribution that was highly skewed in the direction of lower velocities.

[10]Figure 4 also illustrates the modest stimulation of velocity we found with intact caldesmon (2.5 μm/s) compared with control actin–tropomyosin (2.1 μm/s). We have measured similar elevated velocities with low concentrations of calponin (2.6 μm/s). This appears to be due to the elimination of velocity measurement errors under conditions where actin filament binding is enhanced and motion is continuous rather than intermittent. Several observations support this conclusion. We found a similar but smaller increase in velocity with actin–tropomyosin filaments when intermittent motion became more continuous with the addition of methylcellulose to the motility buffer at high salt (80 mM KCl). At low salt (25 mM), where motion appears uninterrupted in the absence of methylcellulose, velocity was not increased in the presence of methylcellulose. And finally, velocity was not increased in the presence of methylcellulose plus the C-terminal fragment of caldesmon where motion is intermittent (2.1–2.2 μm/s, see Fig. 6).

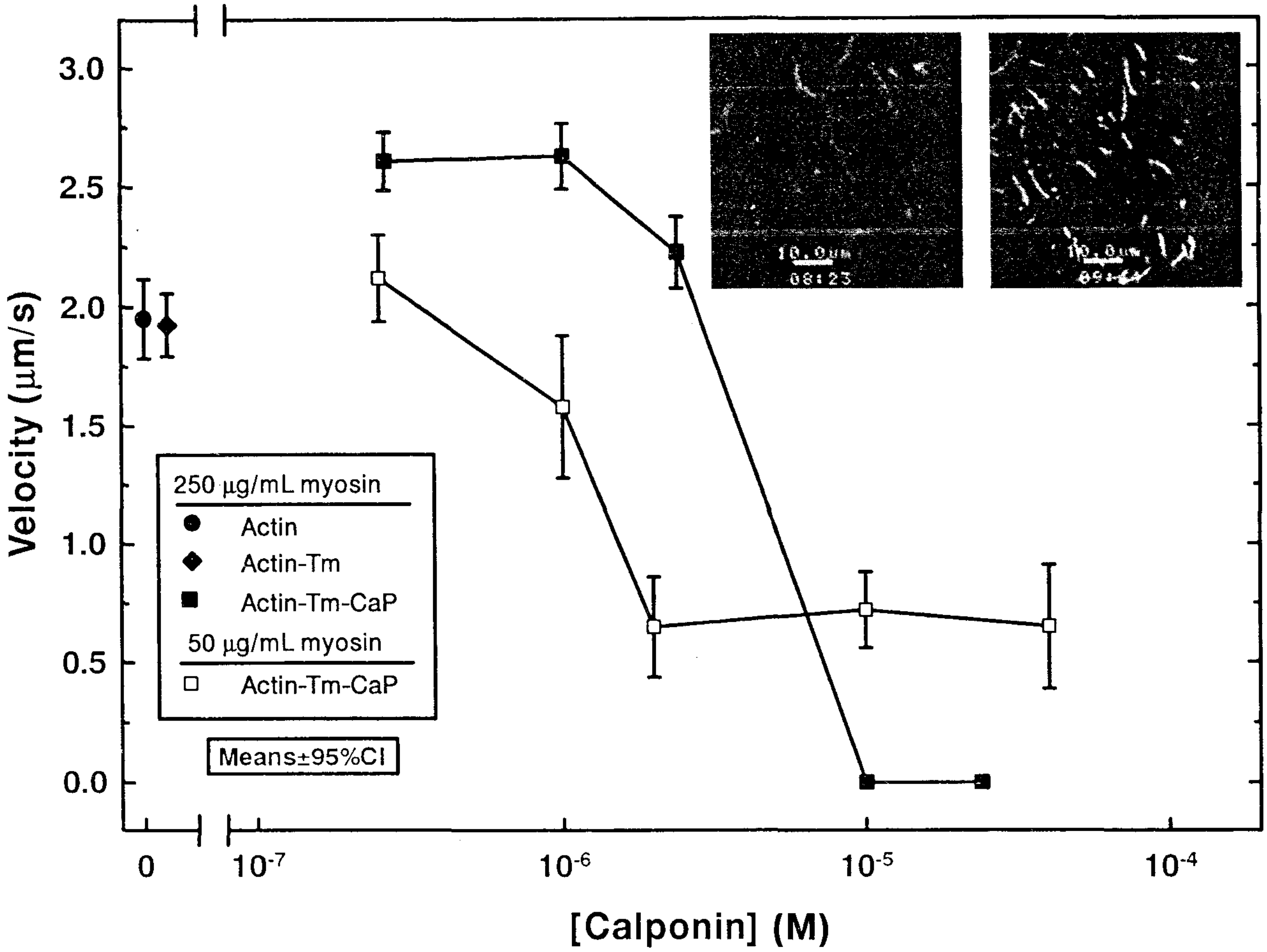


FIG. 7. Effect of calponin (CaP) on filament binding and velocity at high and low myosin densities. Insets panels, effect of calponin on filament binding at high ionic strength. Conditions were the same as for Fig. 4. As shown previously, there was no binding in the absence of methylcellulose and calponin. The addition of 10 μM calponin significantly increased the number of attached filaments, and all filaments remained attached for the duration of the observation period of 1 – 10 min. The plotted data show the effects of calponin on filament velocity. Values are means $\pm$ 95% confidence intervals for at least 10 determinations per point. Adapted from Haeberle (1994).

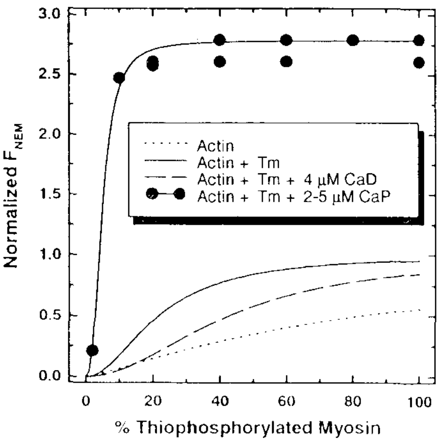


FIG. 8. Effects of tropomyosin (Tm), caldesmon (CaD), and calponin (CaP) on the regulation of $Force_{NEM}$ by LC_{20} phosphorylation. Different levels of myosin thiophosphorylation were achieved by mixing appropriate amounts of dephosphorylated myosin and myosin that had been thiophosphorylated with myosin light chain kinase to 2.0 mol/mol. Values for $Force_{NEM}$ (F_{NEM}) were determined as described in Fig. 2 and by Haeberle (1994). The tropomyosin concentration was 1.8 μM. The actin and actin + Tm curves were fit to the data shown in Fig. 3. All curves are least-squares fits to the Hill equation.

bearing structure that could contribute to the latch state in smooth muscle. It appears more likely that caldesmon-dependent tethering functions to promote the interaction of actin and myosin, as demonstrated in activated platelets (Hemric et al. 1993).

Preliminary findings have shown that caldesmon inhibits $Force_{NEM}$ both in the presence and absence of tropomyosin, with 50% inhibition at 10 μM and complete inhibition at 40 μM in the presence of tropomyosin. As shown in Fig. 8, caldesmon most potentially inhibits the enhancement of $Force_{NEM}$ by tropomyosin at low levels of LC_{20} phosphorylation. If the 25% decrease in velocity that occurs at 10 μM caldesmon (Fig. 4) is due to the loading effect of the caldesmon tether in the face of a 50% reduction of $Force_{NEM}$, and if the curvature of the force – velocity relationship is not affected, then the load imposed at this concentration of caldesmon could be no more than 10 – 20% of the maximum force measured in the presence of actin – tropomyosin.

Taken together, these motility findings show that caldesmon alters f_{app} but has no effect on g_{app}. These findings are controversial in that others have shown that caldesmon inhibits filament velocity in a similar motility assay (Shirinsky et al. 1992; Okagaki et al. 1991). Ishikawa et al. (1991) reported a stimulation of velocity at low concentrations followed by inhibition at high concentrations. While it is not possible to be certain why such differences were found, we have observed that intact caldesmon will inhibit velocity if sulfhydryl-dependent oligomerization is not prevented by the use of a robust free-radical scavenger system as described above, both before and after caldesmon is added to the motility buffer (Fig. 4).

Effects of calponin on filament motility

We initially observed a very abrupt and complete inhibition of filament velocity at about 1 μM calponin (Fig. 7, ■), similar to previous reports by others (Shirinsky et al. 1992). The complete inhibition of motility appears to be the result of the excessively tight binding of actin to myosin in the presence of calponin (Haeberle et al. 1994). Reducing the myosin concentration to 100 μg/mL (□) resulted in normal motility at reduced velocity (0.7 μm/s) in the presence of calponin; velocity was insensitive to further decreases in myosin concentration down to 10 μg/ml.

Analysis of the effect of calponin on force generation demonstrated that there was a 3-fold increase in $\mathrm{Force}_{\mathrm{NEM}}$ when calponin was present, and a dramatic increase in the sensitivity of activation by LC_{20} phosphorylation (Fig. 8). The steep activation in the presence of calponin and tropomyosin is consistent with the idea that smooth muscle tropomyosin enhances the cooperative switching on of actin filaments by strong-binding cross bridges; in the presence of calponin, the number of strong-binding cross bridges would be increased for any level of LC_{20} phosphorylation. Taken together these findings all suggest that calponin decreases g_{app}, but they cannot rule out a modest affect of calponin on f_{app}.

Model for the coregulation of force and velocity by LC_{20} phosphorylation, tropomyosin, caldesmon, and calponin

Figure 9, summarizes the effects of the four putative regulatory proteins on actin filament motility and binding in the motility assay. These findings are the basis for the assignment of each regulatory protein to the regulation of f_{app} or g_{app}. Of these four regulatory proteins, only calponin regulates g_{app} and unloaded velocity. Tropomyosin, LC_{20} phosphorylation, and caldesmon all appear to affect f_{app} exclusively and, therefore, must either activate contraction or modulate the sensitivity of activation.

The qualitative model depicted in Fig. 10, generally outlines our current working model by which these four regulatory proteins could interact to regulate smooth muscle contraction. The left half of the figure corresponds to fully dephosphorylated (turned on) calponin, and the right half is phosphorylated (turned off) calponin. There is still considerable controversy about the phosphorylation state of calponin in situ; however, one of the clearest predictions of this model is that calponin must be regulated, either by phosphorylation or by some alternative second messenger.

Another interesting aspect of the model is the prediction that both caldesmon and calponin can modulate the sensitivity of activation by LC_{20} phosphorylation. The model predicts, however, that caldesmon and calponin would have opposite effects on sensitivity. The model further predicts that only regulation of calponin would be associated with both a change in sensitivity and a change in unloaded shortening velocity. Therefore, in hog carotid artery smooth muscle, where LC_{20} is transiently phosphorylated and then dephosphorylated in parallel with a decline in unloaded shortening velocity while isometric

	F_{NEM}	Velocity	Binding	On Rate	Off Rate
Tm	+	0	+	+	0
LC_{20}	-	0	?	-	0
CaD	-	0	+/-	-	0
CaP	+	-	+	?	-

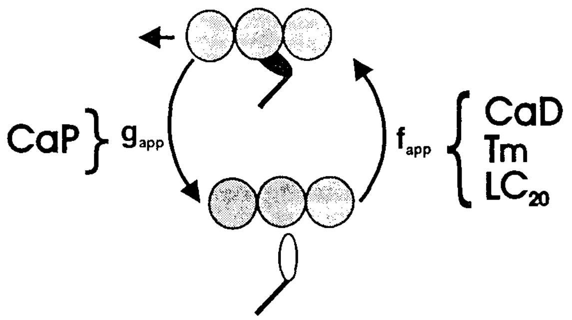


FIG. 9. Summary of the effects of caldesmon (CaD), calponin (CaP), tropomyosin (Tm), and LC_{20} phosphorylation on filament binding, force, velocity, and f_{app} and g_{app}.

force is maintained or increases, the model predicts that calponin must be regulated (phosphorylated and dephosphorylated?) with a time course similar to that for LC_{20} phosphorylation and dephosphorylation.

The model also predicts that constant or increasing isometric force in the face of declining or constant levels of LC_{20} phosphorylation, respectively, must be accompanied by either phosphorylation of caldesmon or activation (dephosphorylation?) of calponin. These two possibilities should be distinguishable on the basis of changes in actin-activated ATPase activity and unloaded shortening velocity. Phosphorylation of caldesmon would have no effect on shortening velocity, but would increase steady-state isometric ATPase activity, whereas activation of calponin would decrease both unloaded velocity and steady-state isometric ATPase activity.

Summary

Primarily on the basis of data derived from an in vitro motility assay, a qualitative model for the coregulation of cross-bridge cycling by myosin light chain phosphorylation, caldesmon, calponin, and tropomyosin has been proposed. In this model, calponin is the sole regulator of the off-rate (g_{app}); caldesmon, tropomyosin, and LC_{20} phosphorylation all modulate the cross-bridge on-rate (f_{app}) and, consequently, govern steady-state force and ATP hydrolysis, with little effect on unloaded velocity. Since only calponin has any effect on g_{app}, activation of the latch state in smooth muscle most likely involves regulation of calponin. In very general terms, this model is consistent with a large portion of the experimental data available, and there are few experimental reports that directly contradict the model. This latter point is more a reflection of the scarcity of phosphorylation data for caldesmon and calponin than it is support for the model. It should also be pointed out that this model does not, in its present form, exclude a more complex scheme resulting from a combination of the present model and any of the alternative models that have been proposed, such as myosin-linked cooperativity (Vyas et al. 1992), modulation of cross-bridge detachment by Mg ADP (Nishimura and van Breeman 1989), rigor-dependent activation of actin (Somlyo et al. 1988), or the latch-state model as described by Hai and Murphy (1988).

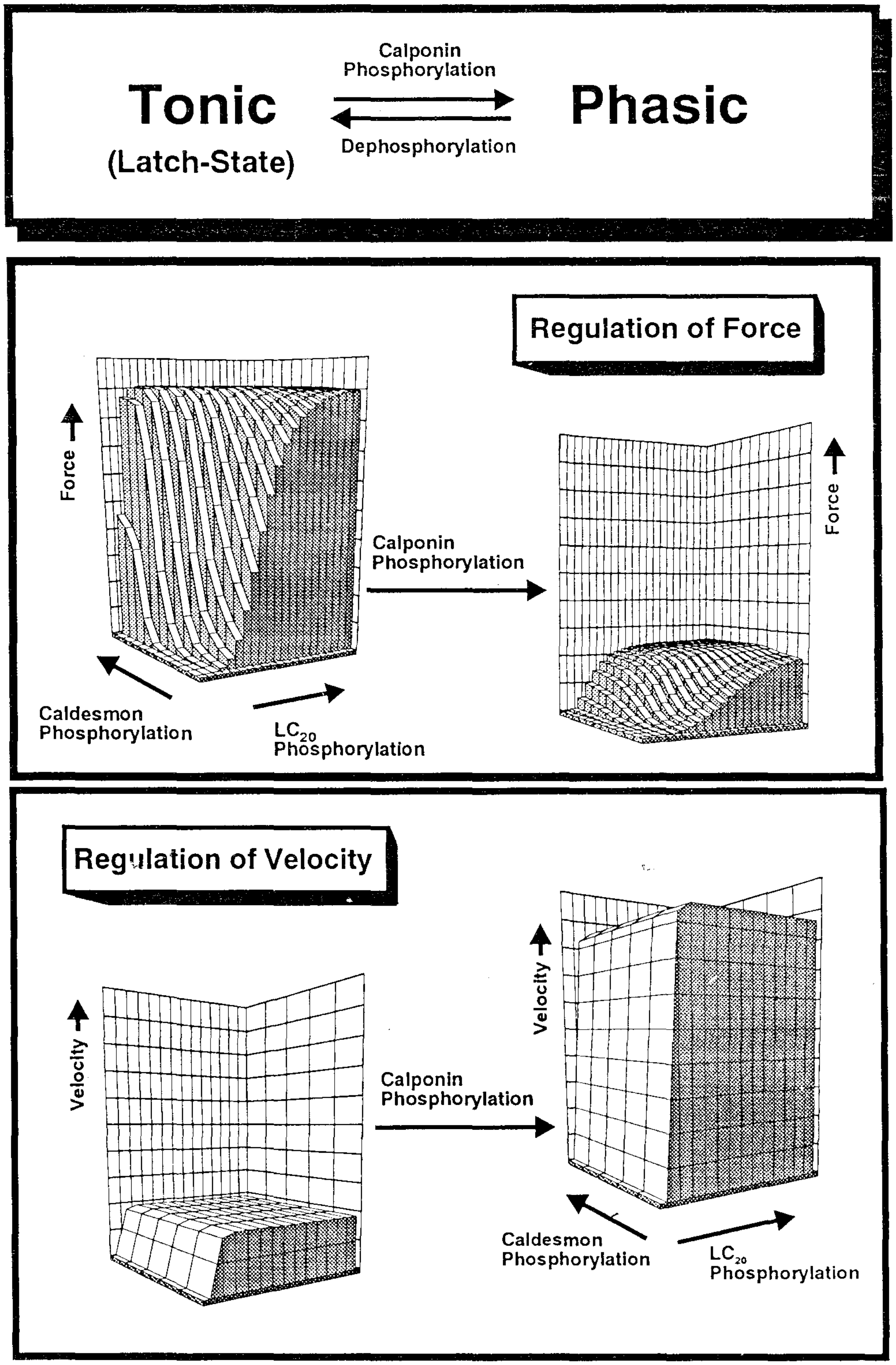


FIG. 10. Model for the combined regulation of smooth muscle contraction by LC_{20}, caldesmon, and calponin.

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