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THE FUNCTION OF MYOSIN AND MYOSIN LIGHT CHAIN KINASE PHOSPHORYLATION IN SMOOTH MUSCLE

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INTRODUCTION

An in-depth understanding of the regulation of contractile tone in smooth muscle takes its form from detailed biochemical descriptions of (*a*) specific effects unique to individual effector inputs at the membrane, (*b*) mechanisms of mediation of the effector signal (second messengers), and (*c*) modes and sites of action of second messengers both within the contractile protein regulatory system and within the membrane-associated homeostatic mechanisms. Most early physiological and pharmacological studies on smooth muscle preparations focused primarily on extracellular effectors and their interactions with the membrane components ultimately involved in the control of intracellular Ca^{2+} concentration. Evaluation of the steady-state contractile response has been interpreted as an index of sarcoplasmic Ca^{2+} concentration, with the underlying assumption that Ca^{2+} activates contractile proteins through a simple switch-like mechanism. The discoveries that the 20,000-dalton light chain of smooth muscle myosin could be phosphorylated (1), and that the phosphorylation of smooth muscle myosin is associated with an increase in its actin-activated Mg^{2+}-ATPase activity (2), have led to a number of biochemical investigations into the regulatory role of the specific phosphorylation of myosin by the Ca^{2+}- and calmodulin-dependent enzyme myosin light chain kinase. Recent investigations have focused on demonstrating the physiological role of myosin phosphorylation in the regulation of contractility in intact smooth muscle.

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This review describes our current understanding of the regulation and role of myosin phosphorylation in smooth muscle contraction and relaxation. It is not historically comprehensive, and we have relied upon reviews by other authors for the citation of certain developments and perspectives. For example, Krebs & Beavo (3) have presented important criteria for establishing that an enzyme undergoes physiologically significant phosphorylation and dephosphorylation. In the context of myosin P-light chain and myosin light chain kinase phosphorylation in smooth muscle, it is worthwhile to review these criteria:

1. Demonstration in vitro that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction(s) catalyzed by an appropriate protein kinase(s) and dephosphorylated by a phosphoprotein phosphatase(s).
2. Demonstration that functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.
3. Demonstration that the enzyme can be phosphorylated and dephosphorylated in vivo or in an intact cell system with accompanying functional changes.
4. Correlation of cellular levels of protein kinase and/or phosphoprotein phosphatase effectors and the extent of phosphorylation of the enzyme.

THE BIOCHEMICAL PROPERTIES OF MYOSIN PHOSPHORYLATION

Smooth Muscle Contractile Proteins

All smooth muscle cells contain the contractile proteins actin, myosin, and tropomyosin (4–6). The enzyme myosin is the primary protein of the thick filament in smooth muscle and is composed of two high molecular–weight subunits, or heavy chains, and two each of two types of low molecular–weight subunits, or light chains. The molecular weight of each heavy chain subunit is about 200,000 daltons, whereas the light chain subunits are 20,000 and 17,000 daltons respectively. The native hexameric form of myosin is configured as an intertwined coiled-tail region embedded in the thick filament and two globular head regions that protrude from the thick filament at regular intervals to form cross bridges. These head regions contain the actin-binding domain, the catalytic site for ATP hydrolysis, and the associated light chain subunits. According to the sliding filament theory of muscle contraction, thick (myosin) and thin (actin and tropomyosin) filaments move past one another. This process is related to the binding of cross bridges to actin and to the hydrolysis of ATP. The sliding filament theory has been developed primarily from detailed investigations of skeletal muscle, but the general organization of thin and thick filaments in smooth muscle is consistent with a similar mechanism of contraction (7–10).

Regulation by Ca^{2+}

The dominant mechanism for Ca^{2+} activation of contractile elements in vertebrate skeletal and cardiac muscle is due to a thin-filament Ca^{2+}-regulatory system, troponin-tropomyosin. The regulation of actin-myosin interactions in smooth muscle by Ca^{2+} is more complex, and different biochemical mechanisms have been proposed. These mechanisms may be differentiated into two general classes involving thick- and thin-filament regulatory processes respectively. In thick-filament regulation, Ca^{2+} binds to calmodulin and the Ca^{2+}-calmodulin complex subsequently binds to and activates myosin light chain kinase (11). Activation of this protein kinase results in the phosphorylation of the 20,000-dalton light chain subunit of myosin, the P-light chain, and the stimulation of actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin (4–6). There is also evidence that phosphorylated smooth muscle myosin Mg^{2+}-ATPase activity may be further increased by Ca^{2+}, which may be related to Ca^{2+} binding directly to myosin (12–16).

Ca^{2+} may also regulate actin-myosin interaction in smooth muscle via thin-filament components. Ebashi (17) has proposed that Ca^{2+} activation is mediated by a thin-filament protein complex referred to as leiotonin. Marston (18), on the other hand, has proposed two thin filament–linked regulatory mechanisms. Isolated thin filaments, which are capable of binding Ca^{2+}, activate myosin Mg^{2+}-ATPase activity in a Ca^{2+}-dependent manner. He also found that the phosphorylation of a 21,000-dalton protein component in thin filaments is associated with an increase in the quantity of high affinity Ca^{2+} binding sites on thin filaments and a decrease in the Ca^{2+} concentration required for half-maximal activation of actin-activated myosin Mg^{2+}-ATPase activity.

In summary, the preponderance of biochemical evidence indicates that there may be more than one mechanism for Ca^{2+} regulation of smooth muscle contraction. As will be discussed below, myosin phosphorylation certainly plays a prominent role; however, it may not be the only Ca^{2+}-dependent regulatory mechanism in smooth muscle contraction. Although it is not possible to differentiate the relative importance of these other biochemical mechanisms, physiological and pharmacological investigations indicate that, in addition to the calmodulin-myosin light chain kinase system, there is probably another Ca^{2+} regulatory mechanism acting in smooth muscle contraction.

Myosin Phosphorylation and ATPase Activity

The stoichiometric phosphorylation of a single serine residue of the P-light chain of myosin is preferentially catalyzed by myosin light chain kinase, which requires two protein components for activity (4–6). One component is the ubiquitous low molecular–weight Ca^{2+}-binding protein calmodulin. The other component is the catalytic subunit of myosin light chain kinase, which has

molecular weights ranging from 130,000 to 155,000 in smooth muscle cells from different animal species (19–22). The inactive enzyme exists as a monomer and is activated by the binding of one mol of calmodulin per mol of myosin light chain kinase when Ca^{2+} binds to calmodulin. The general mechanism of myosin light chain kinase activation (23) is similar to the activation scheme proposed for other calmodulin-dependent enzyme systems (24, 25):

$$4\ Ca^{2+} + \text{calmodulin} \overset{K_{Ca}{}^{2+}}{\rightleftharpoons} Ca_4^{2+} \cdot \text{calmodulin} \qquad 1.$$

$$Ca_4^{2+} \cdot \text{calmodulin} + \text{kinase} \overset{K_{CM}}{\rightleftharpoons} Ca_4^{2+} \cdot \text{calmodulin} \cdot \text{kinase} \qquad 2.$$

It is generally accepted that at least three, if not four, Ca^{2+}-binding sites on calmodulin are occupied for the activation of the various calmodulin-dependent enzymes (24, 25). The importance of pathways for the activation and inactivation of calmodulin-activated enzymes has been emphasized in biological systems (26–28). It has been proposed that activation associated with an increase in cytoplasmic Ca^{2+} concentrations is the result of Ca^{2+} binding first to calmodulin, with subsequent binding to and activation of a calmodulin-dependent enzyme. Inactivation due to a decrease in cytoplasmic Ca^{2+} concentrations follows a different pathway, however. The rate of inactivation is about three orders of magnitude faster when Ca^{2+} first dissociates from the $Ca_4^{2+} \cdot$ calmodulin · enzyme complex. This scheme for the activation and inactivation of myosin light chain kinase in vivo is summarized in Figure 1.

Phosphorylation of smooth muscle P-light chain by the $Ca_4^{2+} \cdot$ calmodulin · myosin light chain kinase complex is thought to allow the activation of myosin Mg^{2+}-ATPase activity by actin, whereas dephosphorylated myosin is not activated (4–6). The quantitative relationship between the extent of P-light chain phosphorylation and actin-activated Mg^{2+}-ATPase activity has been shown not to be linear in avian gizzard smooth muscle myosin. Phosphorylation of both heads of myosin is required for the activation of myosin Mg^{2+}-ATPase activity (29-32). Although this relationship between P-light chain phosphorylation and actin-activated Mg^{2+}-ATPase activity has been demonstrated with gizzard smooth muscle myosin, it is not necessarily a universal property of myosin from all types of smooth muscle, particularly mammalian smooth muscle. Other investigators found a linear relationship between the extent of phosphorylation and actin-activated Mg^{2+}-ATPase activity of myosin purified from swine pulmonary artery (14) and bovine stomach muscle (33). Obviously, this is an important point in considering the relationship between P-light chain phosphorylation and smooth muscle contraction.

Fewer studies have been reported on the biochemical properties of myosin P-light chain phosphatases, although phosphoprotein phosphatases have been

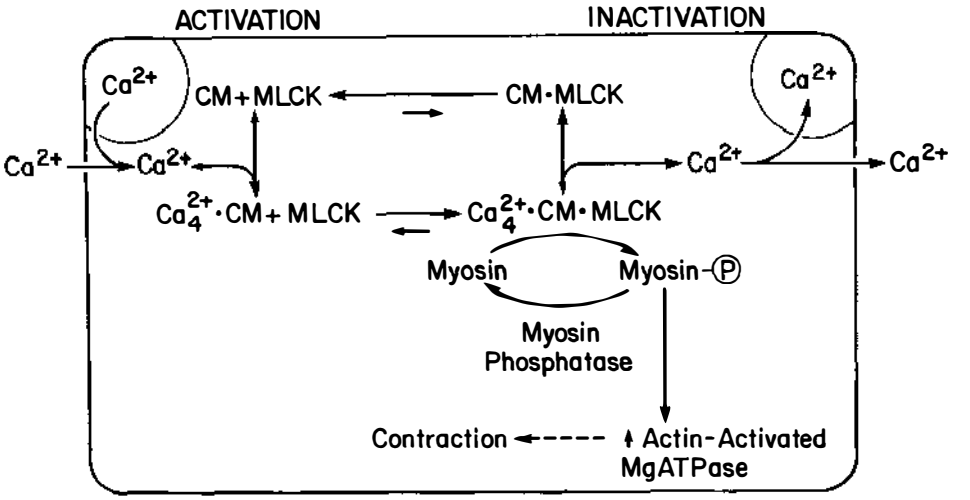

Figure 1 A general scheme for the biochemical regulation of myosin phosphorylation in smooth muscle cells. CM = calmodulin; MLCK = myosin light chain kinase; myosin-Ⓟ = phosphorylated myosin.

purified from smooth muscle (34, 35). A phosphatase that preferentially catalyzes the dephosphorylation of myosin has been purified from bovine aortic smooth muscle (36). Recently, a similar type of phosphatase has been purified from turkey gizzard (37). A preparation of bovine aorta containing actomyosin phosphatase activity has been demonstrated to inhibit the actin-myosin interaction as measured by the Ca^{2+}-dependent superprecipitation of aortic native actomyosin (38). These reports provide evidence of a biochemical mechanism for reversing the effect of myosin phosphorylation by myosin light chain kinase.

The biochemical investigations demonstrating specific stoichiometric phosphorylation of myosin by myosin light chain kinase, dephosphorylation by phosphoprotein phosphatase, and appropriate accompanying activation of myosin Mg^{2+}-ATPase activity satisfy criteria 1 and 2 of Krebs and Beavo mentioned above. These biochemical investigations also provide a framework for understanding the salient features of myosin phosphorylation in smooth muscle cells (Figure 1). In relaxed muscle with low cytoplasmic Ca^{2+} concentrations, little Ca^{2+} is bound to calmodulin, activation of myosin light chain kinase is minimal, and the phosphate content of P-light chain is low. During the initiation of contraction, the concentration of intracellular free Ca^{2+} increases through influx across the sarcolemma and/or through the release of Ca^{2+} from intracellular storage sites. This increase in intracellular Ca^{2+} concentration produces an increase in $Ca^{2+}_4 \cdot$
kinase, which phosphorylates the myosin P-light chain. Phosphorylation re-

sults in an increase in myosin actin–activated Mg^{2+}-ATPase activity. Relaxation caused by the sequestration and removal of Ca^{2+} from the sarcoplasm results in the inactivation of myosin light chain kinase by dissociating Ca^{2+} from the holoenzyme complex. Dephosphorylation of myosin P-light chain by myosin phosphatase then inactivates myosin Mg^{2+}-ATPase activity.

THE REGULATION OF CONTRACTION BY Ca^{2+} AND MYOSIN PHOSPHORYLATION

The combined structural arrangement and enzymatic properties of the contractile proteins within the cell act as a chemomechanical transducer, converting the energy of hydrolysis of ATP to mechanical output. This output, determined by physical constraints on the muscle, can be expressed as force in the isometric case or force and shortening in the isotonic case. In the sliding filament theory based on extensive studies with skeletal muscle, developed force is attributed to the number of active cross bridges generating force additively, this number being regulated by the binding of Ca^{2+} to specific regulatory proteins. Shortening velocity is governed by the load against which the muscle shortens. The maximum velocity of shortening, or V_o, is interpreted as a direct reflection of cross-bridge cycling rates and appears to be determined by the isoenzymatic form of myosin in the cell. This has been demonstrated by the linear correlation between actomyosin Mg^{2+}-ATPase activity and V_o measured in skeletal muscles with widely varying speeds of shortening (39). The similar dependence of velocity on load observed in smooth muscle preparations indicates that the same basic sliding filament mechanism is shared by both muscle types (40).

The dependence of contractile force on elevated levels of calcium in the sarcoplasm surrounding the myofilaments of smooth muscle was originally demonstrated using permeabilized smooth muscle preparations that developed force as a function of calcium concentration in the presence of Mg^{2+}-ATP (41–44). Following the discovery that smooth muscle actin-activated myosin Mg^{2+}-ATPase activity increases upon phosphorylation of P-light chain by Ca^{2+}-calmodulin activated myosin light chain kinase isolated from avian gizzard muscle, more detailed studies with permeabilized or skinned smooth muscle preparations were carried out to demonstrate the significance of this reaction to contractile function. Skinned chicken gizzard preparations showed a correlation between force developed and the degree of P-light chain phosphorylation, although maximum force was produced at 0.2 mol phosphate per mol P-light chain (45). Contractions of a variety of skinned smooth muscle fibers were blocked by specific calmodulin blocking agents (46, 47), and the addition of calmodulin to these preparations enhanced developed force (48, 49). Irreversible thiophosphorylation of myosin resulted in contractions that were maintained in the absence of Ca^{2+} (50, 51). Ca^{2+}-independent contractions

could also be produced by treatment with a Ca^{2+}-insensitive proteolytic fragment of myosin light chain kinase (52). These results are consistent with the notion of a Ca^{2+}- and calmodulin-regulated myosin light chain kinase system activating contraction in smooth muscle.

Stimulus-induced contractions in intact smooth muscle have also demonstrated a major role for myosin phosphorylation in the regulation of contractility; however, at the same time, studies in living cells have revealed the existence of a second Ca^{2+}-dependent regulatory event involved in contraction.

Cellular Myosin Phosphorylation and Dephosphorylation

The significant involvement of myosin phosphorylation in contractile function of intact cells has been demonstrated in studies with a variety of smooth muscle preparations in which different stimuli elicited a net increase in the phosphate content of the P-light chain (Table 1). In all cases, myosin was phosphorylated to levels above basal values during the isometric contraction. Decreases in the extent of P-light chain phosphorylation have been measured during relaxations induced by the removal of the stimulus (53–59), addition of a pharmacological antagonist (60, 61), and treatment with a relaxant agent (61–64). In bovine trachealis, the extent of both phosphorylation and force development was depressed by brief pretreatment with the β-adrenergic agonist isoproterenol (65) and the calmodulin antagonist fluphenazine (58). Thus, smooth muscle cells develop force with the phosphorylation of the contractile protein myosin.

As expected for a Ca^{2+}-dependent enzymatic process, resting values of myosin phosphorylation are generally reported to be low, averaging 0.10 mol phosphate per mol P-light chain (55, 57, 58, 66–68). A few studies have reported resting levels ranging from three to five times this value (69,70). Following protein extraction from quick-frozen tissues, the fraction of phosphorylated and non-phosphorylated P-light chain has been quantitated by determining the relative staining intensities of the two forms after separation during isoelectric focusing (53, 60, 71, 72). Erroneously high values can result from analytical overestimates, which occur if the unphosphorylated P-light chain undergoes a charge modification during electrophoresis so that it comigrates with the phosphorylated form (68). Higher resting values of myosin phosphorylation might also be attributable to differences among tissue preparations, reflecting some degree of tonic contractile activity, unphysiological stress, or possibly cell damage.

Transients in Myosin Phosphorylation

The hypothesis that P-light chain phosphorylation plays a direct role in determining isometric force by activating a certain number of cross bridges has been complicated by the observation that phosphorylation can decline in time while force is maintained (53). The correlation between P-light chain phosphoryla-

Table 1 Maximum net increase in phosphate content of myosin P-light chain in stimulated smooth muscle[a]

Tissue	Stimulus[b]	mol phosphate / mol P-light chain	Stimulus duration	Animal	Reference
Carotid artery	5 μM histamine	0.59	30 seconds	pig	67
	110 mM KCl	0.37	30 seconds	pig	67
	50 μM norepinephrine	0.27	9 minutes	pig	62
	100 mM KCl	0.36	9 minutes	pig	62
Portal vein	"high" KCl	0.68	2–15 seconds	rabbit	66
Trachealis	1 μM carbachol	0.68	60 seconds	steer	65
	60 mM KCl	0.48	60 seconds	steer	65
	electrical (neural)	0.59	5 seconds	steer	59
	100 μM methacholine	0.33	30 seconds	dog	69
	10 μM carbachol	0.33	30 seconds	rabbit	55
Uterus	100 μM carbachol[c]	0.37	20 seconds	rat	63
	0.25 μM $PGF_{2\alpha}$	0.40	30 seconds	rat	64
	100 mM KCl	0.50	30 seconds	rat	68
Taenia coli	electrical[d]	0.22	5 seconds	rabbit	57

[a] Net values were calculated as the difference between the maximum reported averages for each stimulus and the average resting levels of P-light chain phosphorylation in the study.

[b] All studies were in physiological salt solutions containing 1.6–2.5 mM Ca^{2+}. Tissues were incubated at 36–37°C except those noted below.

[c] Incubated at 28°C.

[d] Incubated at 18°C.

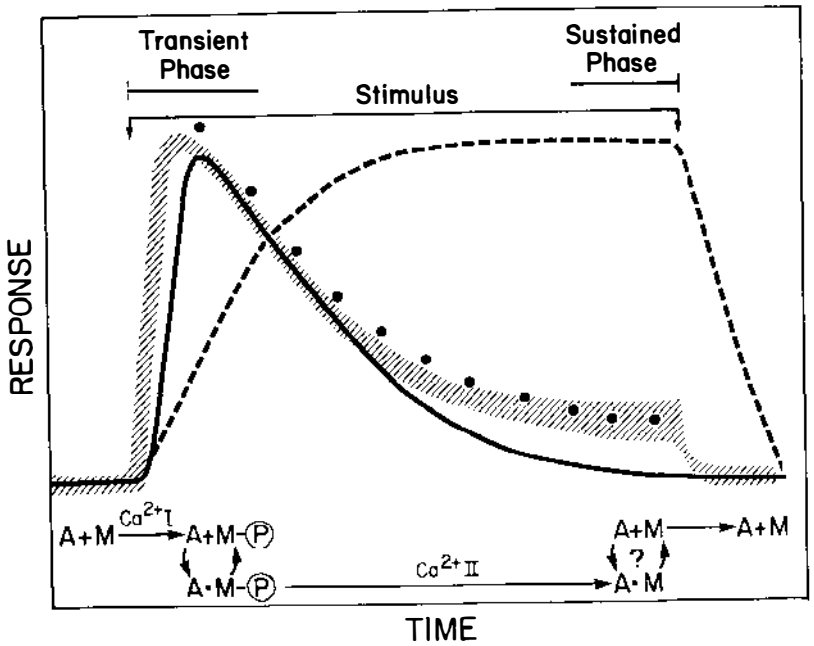

Figure 2 Schematic representative of the processes involved in smooth muscle contraction. The stimulation of resting smooth muscle results in a rapid increase in $Ca^{2+}{}_{cell}$ (shaded profile), which may be transient. The first regulatory site (Ca^{2+}I) for initiating the cyclic interaction of myosin (M) with actin (A) is the Ca^{2+}_4 · calmodulin activation of myosin light chain kinase, which results in P-light chain phosphorylation (— and M-Ⓟ). Attached cross bridges (A•M Ⓟ or A•M) may be rapidly cycling when phosphorylated, resulting in force development (- - -) and high maximal shortening velocities (•), or non- or slowly cycling when dephosphorylated. Whether the low, but measurable, shortening velocities during the sustained phase of contraction reflect the cycling of non-phosphorylated cross bridges or the cycling of a small population of phosphorylated cross bridges is unknown. The second regulatory site (Ca^{2+}II) for force maintenance in the absence of myosin phosphorylation remains unidentified.

tion and isometric force has been shown to vary significantly with both contraction duration and agonist used (65, 67, 73). In the continuous presence of stimuli that maintain constant levels of isometric force, phosphorylation of the myosin P-light chain has been shown to decrease from high initial to significantly lower values in the hog carotid artery (54, 67, 74), bovine (65) and rabbit (55) trachealis, and rabbit taenia coli (57). In the carbachol-stimulated bovine trachealis, Ca^{2+}-dependent phosphorylase *a* formation, like myosin phosphorylation, has been seen to decline with time (65). Thus, the transient increase and decrease in myosin phosphorylation indicates that phosphorylation of myosin may not be the sole Ca^{2+}-dependent regulatory event in smooth muscle contraction (Figure 2).

That the phosphorylation and dephosphorylation of myosin may be related to

changes in sarcoplasmic Ca^{2+} concentrations is indicated by the concomitant increase and decrease in phosphorylase *a* formation, which depends on Ca^{2+} and calmodulin activation of phosphorylase kinase (65, 73). Detailed studies by Murphy and co-workers (67) have confirmed the hypothesis that transients in cell $[Ca^{2+}]$ can account for the phosphorylation transients and have indirectly ruled out or relegated to a minor role three other possible causes: (*a*) reduced substrate (ATP depletion), (*b*) altered access of activated myosin to the kinase or phosphatase, and (*c*) reduced kinase activity secondary to its phosphorylation by cAMP-dependent protein kinase. Agonist-stimulated transients in luminescence by aequorin-loaded vascular cells have indicated that sarcoplasmic Ca^{2+} concentration may indeed vary during maintained force (75). The dependence of isometric force on sarcoplasmic Ca^{2+} concentration is illustrated in the KCl-depolarized hog carotid artery where, after myosin is dephosphorylated to near basal levels, the lowering of extracellular $CaCl_2$ concentration results in a decrease in contractile force (76). It has been hypothesized that the transient increase in myosin phosphorylation is explained by a transient rise in cell Ca^{2+} concentration and that a second regulatory site, responsible for Ca^{2+}-dependent force maintenance, necessarily has a greater Ca^{2+} sensitivity than the activation of myosin light chain kinase by $Ca_4^{2+} \cdot$ calmodulin (67).

It should be emphasized that myosin light chain phosphorylation need not always decline from high to very low values during smooth muscle contraction. The magnitude and rate of phosphorylation measured in tissues varies for both experimental and biological reasons. The time course of transients is affected by experimental temperature. Tissue thickness greatly influences agonist diffusion times. Analysis of tissues frozen soon after stimulation by adding agonist to the bathing medium reflects values in only an outer few activated cells. The true time course of cellular transients may be masked as levels of myosin phosphorylation in cells at different states of activation are averaged. This has been demonstrated by comparing rates of P-light chain phosphorylation seen with transmural electrical stimulation of the cholinergic nerves in the bovine trachealis, where activation is more synchronous than that produced by the muscarinic agonist carbachol. With neural stimulation, myosin phosphorylation reaches maximum values at five seconds, as opposed to 60 seconds in the carbachol-stimulated muscle (59, 65).

The time course of P-light chain phosphorylation also is strongly influenced by the type of smooth muscle under investigation. The activation properties of both cells and tissues are known to vary substantially depending on membrane electrical properties, the relative content of sarcoplasmic reticulum, tissue conductance and cell coupling, receptor populations and distribution, the content of non-muscle cells (mast cells, autonomic nerves, endothelial cells), and species and anatomical location (77). Any of these factors can have a significant effect on calcium distribution and handling during stimulation.

Within an individual muscle preparation, myosin phosphorylation may or may not decline to basal levels depending on stimulus conditions. P-light chain phosphorylation in the bovine trachealis has been observed during a contraction in 1 μM carbachol to decline from a maximum of 0.75 mol phosphate per mol P-light chain at one minute to resting values after two hours of maintained isometric force (Figure 3). Stimulation with 60 mM KCl, on the other hand, leads to a decline from 0.59 to only 0.40 mol phosphate per mol P-light chain after two hours of maintained force (73). Myosin phosphorylation in hog carotid artery depolarized in 110 mM KCl returns to basal levels from a maximum of 0.45 mol phosphate per mol P-light chain after 15 minutes in solutions containing 1.6 mM $CaCl_2$. In the presence of 7.5 mM $CaCl_2$, phosphate content falls from 0.65 to 0.40 mol phosphate per mol P-light chain after 15 minutes and remains unchanged for 30 minutes (67). Steady-state maintenance of P-light chain phosphorylation during contraction is presumably related to specific agonist effects on Ca^{2+} turnover and to maintaining sarcoplasmic Ca^{2+} concentrations above the threshold for myosin light chain kinase activation.

Transients in Mechanical Behavior and Energy Consumption

A more mechanistic description of the involvement of myosin phosphorylation in contractile regulation was proposed after the discovery in arterial muscle that, in parallel with phosphorylation, isotonic shortening velocity against light loads increases during the early phase of contraction and then declines while

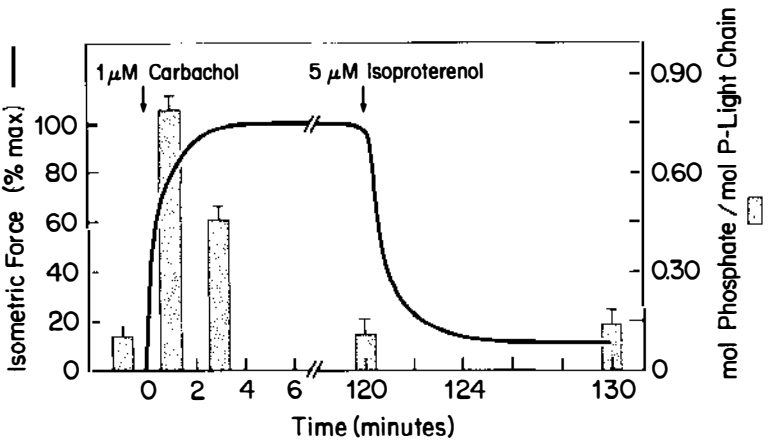

Figure 3 Phosphorylation of myosin P-light chain in bovine trachealis smooth muscle. Muscle strips were contracted by the addition of 1 μM carbachol; after two hours, 5 μM isoproterenol was added. The extent of myosin P-light chain phosphorylation was the same at both 120 and 130 minutes as that in control muscles [adapted from (22)].

force rises to a maintained maximum (74). Levels of myosin phosphorylation during maintained contractions correlate directly with V_o (cross-bridge cycling rate), and not necessarily with absolute levels of stress (number of activated cross bridges) (54). The temporal correlation between myosin phosphorylation and isotonic shortening velocity has since been observed in carbachol-stimulated rabbit trachealis (55) and neurally stimulated bovine trachealis (59). These observations have led to the hypothesis that the Ca^{2+}-dependent phosphorylation of myosin initiates rapid cycling of cross bridges, resulting in the development of isometric force, and that in the presence of a sarcoplasmic Ca^{2+} concentration sufficient to maintain force the dephosphorylation of myosin results in an attached non- or slowly cycling cross bridge (latch bridge). A second Ca^{2+}-dependent mechanism with a greater sensitivity to Ca^{2+} would operate in force maintenance when myosin is dephosphorylated. The dependence of V_o on levels of light chain phosphorylation has been interpreted to be the result of an increase in internal load due to the formation of latch bridges (76). However, a direct modulation of cross-bridge cycling rates by interacting processes activating the myosin molecules has not been ruled out (see above). Maximum velocity of shortening has been observed to be dependent on Ca^{2+} and calmodulin concentrations in skinned preparations (78–80) and on extracellular Ca^{2+} concentration in intact preparations of smooth muscle (67, 80a). While the primary Ca^{2+}-dependent regulatory event appears to be phosphorylation of the myosin P-light chain, there is evidence in intact taenia coli muscle to suggest that Ca^{2+} may have the potential to alter cross-bridge cycling rates while the level of myosin phosphorylation remains constant (80a).

Although force can be maintained in the absence of phosphorylation, these two Ca^{2+}-dependent mechanisms do not appear to be totally independent. Levels of steady-state force have been correlated to initial maximal values of P-light chain phosphorylation (73), and it has been proposed that myosin phosphorylation must precede the latch state. Studies of skinned carotid arteries have demonstrated that, while no difference in Ca^{2+} sensitivity for myosin phosphorylation and force is apparent in the development of contractions, a subsequent reduction in Ca^{2+} concentration results in a proportionally greater dephosphorylation of myosin than reduction in contractile force (81). Thus, myosin phosphorylation may be required for expression of the second Ca^{2+}-dependent mechanism involved in force maintenance during the latch state.

Mechanical (82, 83) and energetic (57) evidence also suggests the existence of a Ca^{2+}-dependent cross-bridge attachment in relaxed or relaxing smooth muscle. Following the removal of a stimulus, P-light chain phosphorylation falls rapidly to resting levels, while force declines at a slower rate (53–57, 59). The Ca^{2+} dependence on force maintenance by dephosphorylated cross bridges during relaxation has been inferred from the acceleration of relaxation by agents believed or known to lower intracellular Ca^{2+} concentration (84).

The decline in cross-bridge cycling rates during stimulation is reflected in measurements of energy consumption in contracting smooth muscle (57, 80a, 85, 86). Such studies emphasize the physiological importance of the latch state, which provides a hypothetical mechanism whereby tonic force can be maintained more efficiently by reducing the ATP consumption associated with high cross-bridge cycling rates and, to a lesser degree, by reducing phosphate turnover in the P-light chain of myosin.

The studies reviewed here fulfill the third criterion of Krebs & Beavo (3) mentioned above, which requires that concomitant functional changes in intact cells be associated with changes in enzyme phosphorylation and dephosphorylation. A simplified scheme representing processes involved with the initiation and maintenance of contractile force in smooth muscle is shown in Figure 2.

Correlation of Cellular Kinase Contents and Activities with Myosin Phosphorylation in Vivo

According to Krebs & Beavo's fourth criterion above (3), the amounts and catalytic properties of the kinase and phosphatase enzymes in the cells should be sufficient to account for the rates and extent of enzyme phosphorylation and its attendant functional alterations. Myosin light chain kinase concentration in tracheal smooth muscle homogenates has been estimated to be 0.36 μM. Assuming the maximal rate of P-light chain phosphorylation by tracheal myosin light chain kinase at 37°C to be 30 μmol phosphate incorporated per liter of intracellular water per second (87) and the rate constant for dephosphorylation to be 0.26 second $^{-1}$ (59), maximal activation of cellular kinase will result in the incorporation of 0.65 mol phosphate per mol P-light chain after two seconds (59). The calculated maximum rate is slightly faster than that measured in the neurally stimulated tracheal smooth muscle (59) and an order of magnitude faster than measured in carbachol-stimulated trachealis (65), demonstrating that maximal rates of enzyme activation and subsequent myosin phosphorylation are not seen in tissues stimulated by externally applied agonists. The time course of P-light chain phosphorylation in the neurally stimulated smooth muscle precedes that of isometric force and coincides with the maximum velocity of shortening.

THE REGULATION OF SMOOTH MUSCLE CONTRACTION-RELAXATION BY CYCLIC AMP

Catecholamines, neurotransmitters, and many peptide hormones increase cyclic AMP formation in smooth muscle tissues by stimulating specific membrane receptors. In particular, cyclic AMP formation has been tightly linked to β-adrenergic receptors in a variety of cells and tissues, and it has been proposed that smooth muscle relaxation via stimulation of β-adrenergic receptors is

mediated by cyclic AMP (88–91). In general, there is a correlation between elevated cyclic AMP content and β-adrenergic induced smooth muscle relaxation or inhibition of contraction. Isoproterenol, a β-adrenergic agonist, inhibits the rate and extent of both tension development and P-light chain phosphorylation in smooth muscle (65). However, these correlations cannot be interpreted as convincing evidence of a role for cyclic AMP in mediating smooth muscle relaxation until the precise biochemical mechanism(s) for the relaxation response has been defined. All known effects of cyclic AMP in mammalian biological systems are thought to be due to cyclic AMP activation of cyclic AMP–dependent protein kinase (3). Therefore, it is logical to propose that the relaxation of smooth muscle by β–adrenergic receptor stimulation may be mediated through the cyclic AMP activation of cyclic AMP–dependent protein kinase as well as the phosphorylation of a key protein(s) involved in the contractile process.

Decreased Sarcoplasmic Ca^{2+} Concentrations

Ca^{2+} plays a central role in eliciting contractions in smooth muscle. Therefore, investigations of mechanisms by which β-adrenergic receptor stimulation may mediate smooth muscle relaxation via cyclic AMP formation have focused on processes involved in excitation-contraction coupling. It has been proposed that cyclic AMP formation may lead to a decrease in Ca^{2+} availability to contractile proteins (88, 91). It is obvious in considering Equations 1 and 2 and Figure 1 that a decrease in Ca^{2+} concentrations in smooth muscle sarcoplasm inhibits myosin P-light chain phosphorylation by decreasing the fractional activation of myosin light chain kinase. The primary mechanisms by which β–adrenergic receptor stimulation may decrease sarcoplasmic Ca^{2+} concentrations include: (*a*) increased Ca^{2+} sequestration into intracellular storage sites (92–95); (*b*) decreased Ca^{2+} influx into smooth muscle cells (96); (*c*) increased Ca^{2+} efflux from smooth muscle cells (97, 98). Regarding the last mechanism, Scheid et al (99) have shown that isoproterenol activates Na^+-K^+ pumping in isolated smooth muscle cells from the toad *Bufo marinus*. These investigators also have shown that membrane fragments from these smooth muscle cells contain Na^+-K^+–dependent ATPase activity stimulated upon incubation with cyclic AMP–dependent protein kinase. These data, as well as the recent demonstration of β-adrenergic stimulation of K^+ influx and Ca^{2+} efflux in smooth muscle cells (98, 100), indicate that relaxation may be mediated in part by increasing Na^+-K^+ pump activity, with a concomitant decrease in contractility via increased Ca^{2+} extrusion by the Na^+-Ca^{2+} exchange mechanism.

Thus, published evidence indicates that β-adrenergic stimulation may decrease sarcoplasmic Ca^{2+} concentrations through three primary mechanisms involved in excitation-contraction coupling. Due to the extensive physiological and pharmacological diversity of smooth muscle cells, it seems likely that the

relative importance of these mechanisms may vary from one type of smooth muscle to another. Additional investigations will be required to elucidate the biochemical mechanisms (presumably due to protein phosphorylation) involved in regulating sarcoplasmic Ca^{2+} concentrations in smooth muscle cells (25).

Myosin Light Chain Kinase Phosphorylation

In 1978, Adelstein et al (101) showed that purified turkey gizzard smooth muscle myosin light chain kinase is phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, which results in a decrease in the rate of P-light chain phosphorylation. Silver and DiSalvo subsequently showed that the addition of cyclic AMP-dependent protein kinase to a crude preparation of bovine aortic actomyosin markedly decreases the Ca^{2+}-dependent phosphorylation of myosin P-light chain (102). Concomitant with the inhibition of myosin P-light chain phosphorylation is a decrease in actin-activated Mg^{2+}-ATPase activity. Based upon these biochemical observations, the intriguing hypothesis was proposed that cyclic AMP may cause smooth muscle relaxation by inhibiting myosin P-light chain phosphorylation via phosphorylation of myosin light chain kinase by cyclic AMP–dependent protein kinase. This hypothesis has been extended by additional investigations on the biochemical properties of this phosphorylation reaction, and recent pharmacological and physiological studies have focused on the properties of myosin light chain kinase phosphorylation in skinned fibers and living smooth muscle cells.

Below we review the criteria of Krebs & Beavo (3) as outlined in the introduction in relation to some recent observations on the phosphorylation of myosin light chain kinase by cyclic AMP–dependent protein kinase.

STOICHIOMETRIC PHOSPHORYLATION Two sites have been phosphorylated in purified turkey gizzard smooth muscle myosin light chain kinase by cyclic AMP–dependent protein kinase (103). In the absence of calmodulin, both sites are phosphorylated, which results in an increase in the concentration of Ca^{2+}_4·calmodulin necessary for 50% activation of myosin light chain kinase activity (K_{CM}; see Equation 2). On the other hand, when calmodulin is bound to myosin light chain kinase, only one site is phosphorylated and this is associated with no change in myosin light chain kinase activity. Analogous studies have also been performed on the dephosphorylation of myosin light chain kinase by a phosphatase purified from turkey gizzard smooth muscle (34). The phosphatase dephosphorylates both sites in myosin light chain kinase in the absence of bound calmodulin. If calmodulin is bound to the diphosphorylated myosin light chain kinase, only one site is readily dephosphorylated, and it coincides with the site that is not phosphorylated by cyclic AMP-dependent protein kinase when calmodulin is bound to myosin light chain kinase.

In general, similar observations have been made on the phosphorylation properties of myosin light chain kinases purified from smooth muscle from bovine stomach (20), porcine myometrium (21), bovine aorta (104), and bovine carotid artery (105). Although myosin light chain kinases purified from bovine cardiac muscle (106) and rabbit skeletal muscle (107) are phosphorylated by cyclic AMP-dependent protein kinase with one mol of phosphate incorporated per mol of myosin light chain kinase, there are no changes in the calmodulin activation properties.

Thus, in terms of Krebs & Beavo's first criterion, it has been shown that two sites in gizzard smooth muscle myosin light chain kinase are stoichiometrically phosphorylated by cyclic AMP–dependent protein kinase. However, the effects of calmodulin on phosphorylation and dephosphorylation reactions require complex consideration. During smooth muscle contraction, calmodulin is bound to myosin light chain kinase (Figure 1). Under these conditions, one would expect a net incorporation of one mol of phosphate per mol of myosin light chain kinase because of inhibition at the other site phosphorylated by cyclic AMP-dependent protein kinase. The dephosphorylation of this site by phosphatase activity is not inhibited by calmodulin. If there were a net incorporation of only 1 mol phosphate per mol myosin light chain kinase in response to an increase in cyclic AMP formation, the calmodulin activation properties would not change.

Activation of myosin light chain kinase by the binding of calmodulin during smooth muscle contraction would also greatly impede the relaxation response due to the slow rate of dissociation of Ca^{2+}_4· calmodulin from myosin light chain kinase. The calculated $t_{1/2}$ for Ca^{2+}_4· calmodulin dissociation is 30–60 minutes. In this regard, it is interesting that previous investigations on skinned fibers from gizzard (108) and tracheal smooth muscle (109) have shown that previously contracted fibers are slowly relaxed over a period of one hour by the addition of the cyclic AMP–dependent protein kinase catalytic subunit.

RATE OF PHOSPHORYLATION At 1 μM myosin light chain kinase, the initial rate of phosphorylation of the turkey gizzard enzyme in the absence of bound calmodulin is 0.2 μmol per minute per mg of cyclic AMP–dependent protein kinase catalytic subunit (103, 107). Purified bovine stomach and tracheal smooth muscle myosin light chain kinase are phosphorylated at initial rates of 0.04 and 0.14 μmol ^{32}P incorporated per minute per mg of cyclic AMP–dependent protein kinase catalytic subunit respectively at 1 μM myosin light chain kinase (20, 110). These concentrations of myosin light chain kinase are close to values thought to exist in smooth muscle (4, 19). Although the values for the kinetic parameters K_m and V_{max} for phosphorylation of smooth muscle myosin light chain kinases are not available, the published rates of phosphorylation may be compared to other physiological protein substrates phosphory-

lated by cyclic AMP–dependent protein kinase. These rates of phosphorylation of myosin light chain kinase are considerably slower than the rates of phosphorylation of phosphorylase kinase (111), pyruvate kinase (112), and glycogen synthase (113). The maximal rates of phosphorylation of these protein substrates range between 18–45 μmol ^{32}P incorporated per minute per mg of cyclic AMP–dependent protein kinase catalytic subunit.

A simple comparison of rates of phosphorylation of purified protein substrates for cyclic AMP–dependent kinase could be misleading in evaluating the importance of a particular phosphorylation reaction in relation to a physiological event (3). It is important to consider the relative amounts of the protein substrate, as well as cyclic AMP–dependent protein kinase, in relation to the physiological event affected. Tissues contain approximately 0.3 μM cyclic AMP–dependent protein kinase in smooth muscle, and the calculated rate of phosphorylation of myosin light chain kinase at 1 μM shows that at least 30 seconds may be required to phosphorylate the two sites in the enzyme in the absence of bound calmodulin, assuming instantaneous maximal activation of cyclic AMP–dependent protein kinase. During contraction, the rate of phosphorylation is considerably slower due to calmodulin binding to myosin light chain kinase.

THE BIOCHEMICAL EFFECT OF PHOSPHORYLATION Conti & Adelstein (103) originally found that phosphorylation of two sites in myosin light chain kinase from turkey gizzard smooth muscle by cyclic AMP–dependent protein kinase results in a 10–20 fold increase in the concentration of Ca^{2+}_4·calmodulin necessary for 50% activation of myosin light chain kinase activity, i.e. a 10–20 fold increase in the K_{CM} value in Equation 2. This effect on the K_{CM} value is reversed if myosin light chain kinase is dephosphorylated by a phosphoprotein phosphatase purified from turkey gizzard smooth muscle. When calmodulin is bound to myosin light chain kinase, only one site is phosphorylated and there is no change in the calmodulin activation properties. It is not clear at this time whether phosphorylation of both sites is required for the change in the calmodulin activation properties or whether a single site may be phosphorylated with a concomitant increase in K_{CM}. Miller et al (22) found that phosphorylation of purified bovine tracheal smooth muscle myosin light chain kinase by cyclic AMP–dependent protein kinase to 2 mol phosphate per mol myosin light chain kinase results in a twelvefold increase in K_{CM}. Similar types of results have been obtained with myosin light chain kinase purified from bovine stomach smooth muscle (20), porcine myometrium (21), bovine aorta (104), and bovine carotid artery (105). Thus, myosin light chain kinase purified from a variety of smooth muscles from different animals is phosphorylated in the presence of cyclic AMP–dependent protein kinase, and phosphorylation in the absence of

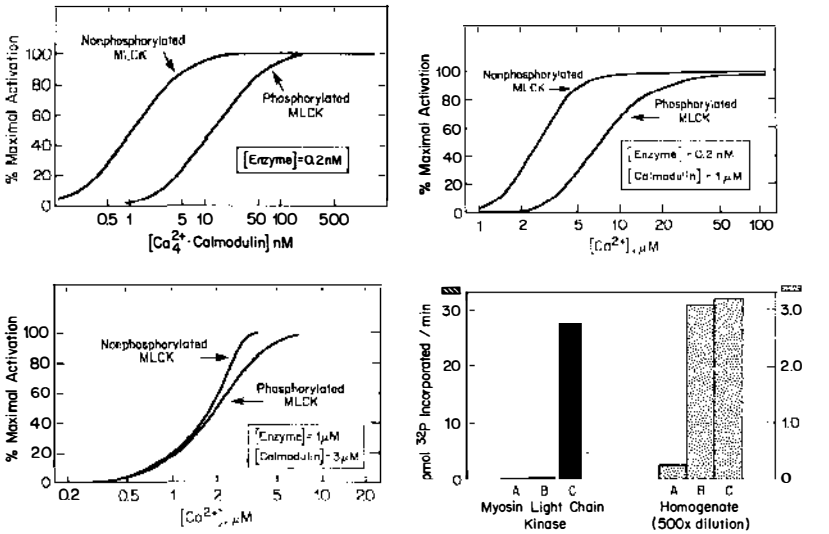

Figure 4 The activation properties of nonphosphorylated and diphosphorylated myosin light chain kinase from smooth muscle. *Upper left:* the effect of Ca_4^{2+}· calmodulin on kinase activities at 0.2 nM myosin light chain kinase; *upper right:* the effect of Ca^{2+} on kinase activities at 0.2 nM myosin light chain kinase and 1 μM calmodulin; *lower left:* the effect of Ca^{2+} on kinase activities at 1 μM myosin light chain kinase and 3 μM calmodulin; *lower right:* the effect of EGTA (A), 200 μM Ca^{2+} (B), or 100 nM calmodulin and 200 μM Ca^{2+} (C) on myosin light chain kinase activity of the purified enzyme (left) and in a homogenate of tracheal smooth muscle (right). Assays were performed according to conditions described in (23).

bound calmodulin results in an increase in the concentration of Ca_4^{2+}· calmodulin required for activation (Figure 4, upper left).

In general, the K_{CM} values change from 1 nM to 10 nM Ca_4^{2+}· calmodulin upon diphosphorylation. These concentrations of calmodulin are considerably lower than the total calmodulin content in smooth muscle, which has been estimated to be 3–6 μM calmodulin (4, 6, 24). However, the prediction may be made from Equations 1 and 2 that a change in the K_{CM} value for myosin light chain kinase would result in a change in the concentration of Ca^{2+} required for activation of myosin light chain kinase in the presence of a high concentration of calmodulin (Figure 4, upper right). At 1 μM calmodulin and low Ca^{2+} concentrations, there is insufficient Ca_4^{2+}· calmodulin for the activation of either the nonphosphorylated or the phosphorylated myosin light chain kinase. As the Ca^{2+} concentration is increased, the concentration of Ca_4^{2+}· calmodulin is increased due to the occupation of the four divalent binding sites on calmodulin with Ca^{2+} (Equation 1). Because diphosphorylated myosin light chain kinase requires a higher concentration of Ca_4^{2+}· calmodulin for activation, Ca^{2+} concentrations required for activation will be greater. Thus, in a biological

setting the high concentration of calmodulin per se does not preclude the possibility that phosphorylation of myosin light chain kinase may be important in the regulation of myosin light chain kinase activity. The primary effect of diphosphorylation of the enzyme would be to increase the concentration of Ca^{2+} required for half-maximal activation in the presence of high concentrations of calmodulin.

However, there is another element that should be considered before these properties are extrapolated to living smooth muscle cells. Measurements of calmodulin or Ca^{2+} activation of myosin light chain kinase (Figure 4, upper left and right) are usually done at concentrations of enzyme that are well below K_{CM}, i.e. approximately 0.2–0.5 nM. The content of myosin light chain kinase in smooth muscle is approximately 0.5–1 μM (4, 19, 20). Thus, concentrations of myosin light chain kinase and calmodulin are both three orders of magnitude greater than K_{CM}, a factor that would have a significant effect upon activation properties (Figure 4, lower left; D. K. Blumenthal, J. T. Stull, unpublished observations). The high concentration of myosin light chain kinase acts as a buffer, so that $Ca_4^{2+}\cdot$ calmodulin formed at low concentrations (nM range) would be bound to myosin light chain kinase. A change in K_{CM} from 1 to 10 nM would result in no significant change in the Ca^{2+} concentrations required for half-maximal activation at high enzyme and calmodulin concentrations (Figure 4, lower left). One way of resolving this dilemma is to invoke competition for $Ca_4^{2+}\cdot$ calmodulin binding to other calmodulin binding proteins, so that the amount of $Ca_4^{2+}\cdot$ calmodulin is not sufficient for full activation of myosin light chain kinase. We have explored this possibility by determining whether calmodulin activation of myosin light chain kinase is limiting in tracheal smooth muscle (Figure 4, lower right). As expected, purified myosin light chain kinase is not activated when Ca^{2+} is added to the reaction mixture and requires calmodulin in addition to Ca^{2+}. Myosin light chain kinase activity in a homogenate prepared from tracheal smooth muscle, on the other hand, is fully activated by the addition of Ca^{2+} and the addition of calmodulin produces no further activation. These results indicate that there is sufficient calmodulin in tracheal smooth muscle to activate fully myosin light chain kinase, and that there are not sufficient amounts of other calmodulin binding proteins to compete effectively with myosin light chain kinase for $Ca_4^{2+}\cdot$ calmodulin.

The effect of cyclic AMP–dependent protein kinase on the calcium sensitivity of actin-myosin interactions has been investigated in a more intact protein system involving skinned bundles of different types of smooth muscles. Kerrick & Hoar (108) were the first to show that the addition of the catalytic subunit of cyclic AMP–dependent protein kinase inhibits Ca^{2+}-activated tension of skinned gizzard smooth muscle fibers. Qualitatively similar results have been obtained with skinned fibers from guinea pig taenia coli (114), hog carotid artery (115), and guinea pig trachealis (109). In most of these studies, the

catalytic subunit of cyclic AMP–dependent protein kinase was preincubated with skinned fibers in the presence of a low Ca^{2+} concentration that did not elicit contraction. Under these conditions, the extent of steady-state contractile force is inhibited with increasing Ca^{2+} concentrations, in a manner similar to the effect of phosphorylation on Ca^{2+} activation of myosin light chain kinase (Figure 4, upper right). Kerrick & Hoar (108) found that the addition of 5 μM calmodulin in the presence of Ca^{2+} reverses the inhibitory effect or prevents relaxation with the subsequent addition of the catalytic subunit. Similar observations have been made by Sparrow et al (109) and Rüegg & Paul (115). Meisheri & Rüegg (116) found cyclic AMP itself relaxes skinned guinea pig taenia coli. At physiological concentrations of calmodulin (5 μM), cyclic AMP–induced relaxation is completely inhibited at low Ca^{2+} concentrations. These investigators proposed that the decrease in sarcoplasmic free Ca^{2+} concentrations by cyclic AMP may be the important determinant for the inhibition of contraction and that a direct inhibitory effect of cyclic AMP on actomyosin interaction (phosphorylation of myosin light chain kinase) plays a secondary role.

PHOSPHORYLATION IN VIVO WITH ACCOMPANYING FUNCTIONAL CHANGES Although no measurements of myosin light chain kinase phosphorylation in vivo have been reported in which the calmodulin binding properties are also analyzed, the potential physiological role for this phosphorylation reaction has been assessed indirectly. Nishikori et al (64) have measured myosin light chain kinase activity from the uteri of estrogen-primed rats stimulated to contract with prostaglandin $F_{2\alpha}$ and then exposed to relaxin. The stimulation of cyclic AMP formation and the relaxation of uteri by relaxin decreases the extent of phosphorylation of myosin light chain and decreases myosin light chain kinase activity. However, these investigators uncovered some unusual features of myosin light chain kinase. About half of the total kinase activity was Ca^{2+}-independent, and it is this activity that primarily decreases in extracts prepared from relaxin-treated uterine smooth muscle. The Ca^{2+}-independent kinase activity may represent partially proteolyzed myosin light chain kinase or another protein kinase. The Ca^{2+} activation properties of the Ca^{2+} and calmodulin-dependent kinase activity (64) are not typical of smooth muscle myosin light chain kinase in that the curves of kinase activity do not show any positive cooperativity (6). The amount of myosin light chain kinase activity extracted from the washed myofibrillar pellets prepared from the uteri is lower by several orders of magnitude than values normally obtained (19, 20). We have found (J. R. Miller, J. T. Stull, unpublished observations) that homogenization of quick-frozen mammalian smooth muscle results in the supernatant fraction rather than the pellet containing most of the myosin light

chain kinase activity. Thus, most of the uterine myosin light chain kinase activity may not have been measured. Because of these problems, it is difficult to reach a definitive conclusion about changes in the enzymatic properties of uterine smooth muscle myosin light chain kinase in response to relaxin.

Miller et al (22) have developed a simple assay for assessing changes in calmodulin activation properties that measures the ratio of myosin light chain kinase activities in the presence of 4 μM–100 μM Ca^{2+} at 1 μM calmodulin. This activity ratio in tracheal muscle strips incubated under control conditions is 0.80, a value identical to non-phosphorylated myosin light chain kinase (Figure 4, upper right). When purified myosin light chain kinase is phosphorylated at both sites by cyclic AMP--dependent protein kinase, there is a decrease in the activity ratio to 0.24, corresponding to a twelve-fold increase in K_{CM}. However, the ratio of myosin light chain kinase activity is unchanged when tracheal smooth muscle is incubated with the β-adrenergic agonist isoproterenol at a concentration sufficient to relax the muscle. These results indicate that there are no changes in the calmodulin activation properties of myosin light chain kinase in tracheal smooth muscle upon β-adrenergic stimulation. Thus, phosphorylation of both sites in myosin light chain kinase with the concomitant increase in K_{CM} may not occur in smooth muscle cells.

As pointed out previously, phosphorylation of smooth muscle myosin P-light chain can be a transient event in relation to force development. It has been found that after prolonged incubation with carbachol the extent of phosphorylation of myosin P-light chain decreases to control values after two hours while force is maintained, and the addition of isoproterenol at this time results in relaxation (Figure 3). Thus, β-adrenergic receptor stimulation may relax tracheal smooth muscle in the absence of the significant phosphorylation of myosin P-light chain. A similar experimental approach has recently been made with hog carotid artery (84). Under conditions where force is maintained and myosin P-light chain phosphorylation is at basal levels, forskolin-stimulated cyclic AMP formation relaxes the muscles with no detectable change in the extent of myosin phosphorylation. Under similar conditions adenosine, 3-isobutyl-1-methylxanthine, sodium nitroprusside, and 8-bromo-cyclic GMP also relax the muscle. Thus, these experiments show that dephosphorylation of myosin is not necessary for relaxation and in particular that agents that increase cyclic AMP formation also relax vascular smooth muscle. Jones et al (117) have measured changes in ion fluxes in relation to relaxation by forskolin contractions produced by norepinephrine, angiotensin II, and KCl depolarization in rat aorta. These authors conclude that cyclic AMP–dependent regulation of membrane ion fluxes represents a primary mechanism for relaxation and that the phosphorylation of myosin light chain kinase apparently functions in a secondary capacity. These pharmacological studies in isolated smooth muscle strips indicate that cyclic AMP--dependent effects on sarcoplasmic Ca^{2+} con-

centrations may be the most important process in mediating smooth muscle relaxation.

CORRELATION OF CYCLIC AMP CONTENT WITH THE EXTENT OF PHOSPHORYLATION IN VIVO De Lanerolle et al (61) measured the extent of radioactive phosphate incorporated into myosin light chain kinase of tracheal smooth muscle and found that the estimated phosphate incorporation under control conditions is 1.1 mol phosphate per mol myosin light chain kinase. Stimulation of cyclic AMP formation with forskolin in the absence or presence of methacholine increases the net phosphate incorporated into myosin light chain kinase by 0.6–0.8 mol phosphate per mol myosin light chain kinase respectively. When forskolin is added in the presence of methacholine, the tracheal smooth muscle relaxes. Under control conditions with low cyclic AMP levels (61) and low phosphorylation of phosphorylase (22, 65), cyclic AMP–dependent protein kinase is probably not activated. Hence, the phosphate incorporated into myosin light chain kinase under these conditions is probably not catalyzed by cyclic AMP–dependent protein kinase. Nonspecific phosphorylation in vivo is a feature common to regulated enzymes (3). During smooth muscle contraction and calmodulin activation of myosin light chain kinase, a net incorporation of 1 mol phosphate per mol myosin light chain kinase in the site that does not result in an increase in K_{CM} would be expected, and these data on the phosphorylation of myosin light chain kinase in intact tracheal smooth muscle are consistent with this notion. There have been no measurements of the calmodulin activation properties of tracheal smooth muscle myosin light chain kinase from control and forskolin-treated strips. Nor have there been any measurements of the sites phosphorylated by established peptide mapping procedures (110). Thus, no investigations with smooth muscles have yet correlated the extent of cyclic AMP formation with the phosphorylation of the two sites phosphorylated in myosin light chain kinase by cyclic AMP–dependent protein kinase.

CONCLUDING REMARKS

The Ca^{2+}-dependent phosphorylation of myosin P-light chain is an important, if not obligatory, regulatory step in the initiation of smooth muscle contraction. While myosin phosphorylation in itself may be sufficient to maintain isometric tension, it is evident that a second, as yet unidentified, Ca^{2+}-dependent regulatory mechanism is capable of operating in the maintenance of contractile force after the dephosphorylation of myosin. The identification and characterization of this second site presents an important area for future investigation.

The phosphorylation of purified smooth muscle myosin light chain kinases by cyclic AMP–dependent protein kinase results in a change in the enzymatic

properties of the enzyme, with an increase in the Ca^{2+}_4· calmodulin concentrations required for activity. This basic observation has been used as a hypothesis for considering an important role for cyclic AMP in mediating smooth muscle relaxation via the phosphorylation of myosin light chain kinase. However, an examination of this intriguing hypothesis in relation to the specific criteria set forth by Krebs & Beavo (3) for establishing the physiological significance of a protein phosphorylation reaction reveals unresolved problems, indicating that it may not be a primary event in mediating smooth muscle relaxation associated with cyclic AMP formation. Observations with living smooth muscles indicate that a decrease in sarcoplasmic Ca^{2+} concentration may be the primary biochemical event that mediates smooth muscle relaxation upon β-adrenergic receptor stimulation.

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Literature Cited

1. Frearson, N., Focant, B. W. W., Perry, S. V. 1976. Phosphorylation of a light chain component of myosin from smooth muscle. *FEBS Lett.* 63:27–32
2. Sobieszek, A. 1977. Vertebrate smooth muscle myosin. Enzymatic and structural properties. In *The Biochemistry of Smooth Muscle*, ed. N. L. Stephens, pp. 413–43. Baltimore: Univ. Park
3. Krebs, E. G., Beavo, J. A. 1979. Phosphorylation-dephosphorylation of enzymes. *Ann. Rev. Biochem.* 48:923–59
4. Adelstein, R. S., Eisenberg, E. 1980. Regulation and kinetics of the actin-myosin-ATP interaction. *Ann. Rev. Biochem.* 49:921–56
5. Hartshorne, D. J., Gorecka, A. 1980. Biochemistry of the contractile proteins of smooth muscle. In *Handbook of Physiology. The Cardiovascular System. Vol. 2: Vascular Smooth Muscle*, ed. D. F. Bohr, A. P. Somlyo, H. V. Sparks Jr., pp. 93–120. Bethesda: Am. Physiol. Soc.
6. Stull, J. T. 1980. Phosphorylation of contractile proteins in relation to muscle function. *Adv. Cyclic Nucleotide Res.* 13:39–93
7. Somlyo, A. V. 1980. Ultrastructure of vascular smooth muscle. See Ref. 5, pp. 33–67
8. Bagby, R. M. 1983. Organization of contractile/cytoskeletal elements. In *Biochemistry of Smooth Muscle*, ed. N. L. Stephens, 1:1–84. Boca Raton: CRC
9. Small, J. V., Sobieszek, A. 1983. Contractile and structural proteins of smooth muscle. See Ref. 8, 1:85–140
10. Gabella, G. 1984. Structural apparatus for force transmission in smooth muscles. *Physiol. Rev.* 64:455–77
11. Dabrowska, R., Aromatorio, D., Sherry, J. M. F., Hartshorne, D. J. 1977. Composition of the myosin light chain kinase from chicken gizzard. *Biochem. Biophys. Res. Commun.* 78:1263–72
12. Chacko, S., Conti, M. A., Adelstein, R. S. 1977. Effect of phosphorylation of smooth muscle myosin on actin activation and Ca^{2+} regulation. *Proc. Natl. Acad. Sci. USA* 74:129–33
13. Rees, D. D., Frederiksen, D. W. 1981. Calcium regulation of porcine aortic myosin. *J. Biol. Chem.* 256:357–64
14. Chacko, S., Rosenfeld, A. 1982. Regulation of actin-activated ATP hydrolysis by arterial myosin. *Proc. Natl. Acad. Sci. USA* 79: 292–96
15. Nag, S., Seidel, J. C. 1983. Dependence on Ca^{2+} and tropomyosin of the actin-activated ATPase activity of phosphorylated gizzard myosin in the presence of low concentrations of Mg^{2+}. *J. Biol. Chem.* 258:6444–49
16. Kaminski, E. A., Chacko, S. 1984. Effects of Ca^{2+} and Mg^{2+} on the actin-

activated ATP hydrolysis by phosphorylated heavy meromyosin from arterial smooth muscle. *J. Biol. Chem.* 259: 9104–8
17. Ebashi, S. 1983. Regulation of contractility. In *Muscle and Nonmuscle Motility*, ed. A. Stracher, 1:217–32. New York: Academic
18. Marston, S. B. 1982. The regulation of smooth muscle contractile proteins. *Prog. Biophys. Mol. Biol.* 41:1–41
19. Adelstein, R. A., Klee, C. B. 1981. Purification and characterization of smooth muscle myosin light chain kinase. *J. Biol. Chem.* 256:7501–9
20. Walsh, M. P., Hinkins, S., Flink, I. L., Hartshorne, D. J. 1982. Bovine stomach myosin light chain kinase: Purification, characterization, and comparison with the turkey gizzard enzyme. *Biochemistry* 21:6890–96
21. Higashi, K., Fukunaga, K., Matsui, K., Maeyama, M., Miyamoto, E. 1983. Purification and characterization of myosin light-chain kinase from porcine myometrium and its phosphorylation and modulation by cyclic AMP–dependent protein kinase. *Biochim. Biophys. Acta* 747:232–40
22. Miller, J. R., Silver, P. J., Stull, J. T. 1983. The role of myosin light chain kinase phosphorylation in β-adrenergic relaxation of tracheal smooth muscle. *Mol. Pharmacol.* 24:235–42
23. Blumenthal, D. K., Stull, J. T. 1980. Activation of skeletal muscle myosin light chain kinase by Ca^{2+} and calmodulin. *Biochemistry* 19:5608–14
24. Klee, C. B., Crouch, T. H., Richman, P. G. 1980. Calmodulin. *Ann. Rev. Biochem.* 49:489–515
25. Rasmussen, H., Barrett, P. Q. 1984. Calcium messenger system: An integrated view. *Physiol. Rev.* 64:938–84
26. Cox, J. A., Malnoe, A., Stein, E. A. 1981. Regulation of brain cyclic nucleotide phosphodiesterase by calmodulin. A quantitative analysis. *J. Biol. Chem.* 256:3218–22
27. Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., Sharma, R. K. 1981. Mechanism of activation of cyclic nucleotide phosphodiesterase: Requirement of the binding of four Ca^{2+} to calmodulin for activation. *Proc. Natl. Acad. Sci. USA* 78:871–74
28. Chau, V., Huang, C. Y., Chock, P. B., Wang, J. H., Sharma, R. 1982. Kinetic studies of the activation of cyclic nucleotide phosphodiesterase by Ca^{++} and calmodulin. In *Calmodulin and Intracellular Ca^{++} Receptors*, ed. S. Kakiuchi, H. Hidaka, A. R. Means, pp. 199–217. New York: Plenum
29. Ikebe, M., Ogihara, S., Tonomura, Y. 1982. Nonlinear dependence of actin-activated Mg^{2+}-ATPase activity on the extent of phosphorylation of gizzard myosin and H-mcromyosin. *J. Biochem.* 91:1809–12
30. Persechini, A., Hartshorne, D. J. 1981. Phosphorylation of smooth muscle myosin: Evidence for cooperativity between the myosin heads. *Science* 213: 1383–85
31. Persechini, A., Hartshorne, D. J. 1983. Ordered phosphorylation of the two 20,000 molecular weight light chains of smooth muscle myosin. *Biochemistry* 22:470–76
32. Sellers, J. R., Chock, P. B., Adelstein, R. S. 1983. The apparently negatively cooperative phosphorylation of smooth muscle myosin at low ionic strength is related to its filamentous state. *J. Biol. Chem.* 258:14181–88
33. Chacko, S. 1981. Effects of phosphorylation, calcium ion, and tropomyosin on actin-activated adenosine 5'-triphosphatase activity of mammalian smooth muscle myosin. *Biochemistry* 20:702–7
34. Pato, M. D., Adelstein, R. S. 1983. Purification and characterization of a multisubunit phosphatase from turkey gizzard smooth muscle. The effect of calmodulin binding to myosin light chain kinase on dephosphorylation. *J. Biol. Chem.* 258: 7047–54
35. Pato, M. D., Adelstein, R. S. 1983. Characterization of a Mg^{2+}-dependent phosphatase from turkey gizzard smooth muscle. *J. Biol. Chem.* 258:7055–58
36. Werth, D. K., Haeberle, J. R., Hathaway, D. R. 1982. Purification of a myosin phosphatase from bovine aortic smooth muscle. *J. Biol. Chem.* 257: 7306–9
37. Pato, M. D., Kerc, E. 1984. Purification and characterization of smooth muscle phosphatase-IV from turkey gizzard. *Biophys. J.* 45:354 (Abstr.)
38. DiSalvo, J., Gifford, D., Bialojan, C., Rüegg, J. C. 1983. An aortic spontaneously active phosphatase dephosphorylates myosin and inhibits actin-myosin interaction. *Biochem. Biophys. Res. Commun.* 111:906–11
39. Bárány, M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. *J. Gen. Physiol.* 50:197–218
40. Murphy, R. A. 1980. Mechanics of vascular smooth muscle. See Ref. 5, pp. 325–51

41. Filo, R. S., Bohr, D. F., Rüegg, J. C. 1965. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. *Science* 147:1581–83
42. Endo, M., Kitazawa, T., Yagi, S., Lino, M., Kakata, Y. 1977. Some properties of chemically skinned smooth muscle fibers. In *Excitation-Contraction Coupling in Smooth Muscle*, ed. R. Casteels, T. Godfraind, J. C. Rüegg, pp. 199–209. Amsterdam: Elsevier/North Holland Biomed.
43. Gordon, A. R. 1978. Contraction of detergent-treated smooth muscle. *Proc. Natl. Acad. Sci. USA* 75:3527–30
44. Saida, K., Nonomura, Y. 1978. Characteristics of Ca^{2+}- and Mg^{2+}-induced tension development in chemically skinned smooth muscle fibers. *J. Gen. Physiol.* 72:1–14
45. Kerrick, W. G. L., Hoar, P. E., Cassidy, P. S. 1980. Calcium-activated tension: The role of myosin light chain phosphorylation. *Fed. Proc.* 39:1558–63
46. Kerrick, W. G. L., Hoar, P. E., Cassidy, P. S., Bolles, L., Malencik, D. A. 1981. Calcium-regulatory mechanisms: Functional clarification using skinned fibers. *J. Gen. Physiol.* 77:177–90
47. Kreye, V. A. W., Rüegg, J. C., Hofmann, F. 1983. Effect of calcium-antagonist and calmodulin-antagonist drugs on calmodulin-dependent contractions of chemically skinned vascular smooth muscle from rabbit renal arteries. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 323:85–89
48. Cassidy, P., Kerrick, W. G. L., Hoar, P. E., Malencik, D. A. 1981. Exogenous calmodulin increases Ca^{2+} sensitivity of isometric tension, activation and myosin phosphorylation in skinned smooth muscle. *Pflügers Arch.* 392:115–20
49. Sparrow, M. P., Mrwa, U., Hoffman, F., Rüegg, J. C. 1981. Calmodulin is essential for smooth muscle contraction. *FEBS Lett.* 125:141–45
50. Hoar, P. E., Kerrick, W. G. L., Cassidy, P. S. 1979. Chicken gizzard: Relation between calcium activated phosphorylation and contraction. *Science* 204:503–6
51. Peterson, J. W. III. 1982. Simple model of smooth muscle myosin phosphorylation and dephosphorylation as rate-limiting mechanism. *Biophys. J.* 37:453–59
52. Walsh, M. P., Bridenbaugh, R., Hartshorne, D. J., Kerrick, W. G. L. 1982. Phosphorylation-dependent activated tension in skinned gizzard muscle fibers in the absence of Ca^{2+}. *J. Biol. Chem.* 257:5987–90
53. Driska, S. P., Aksoy, M. O., Murphy, R. A. 1981. Myosin light chain phosphorylation associated with contraction in arterial smooth muscle. *Am. J. Physiol.* 240:C222–33
54. Aksoy, M. O., Murphy, R. A., Kamm, K. E. 1982. Role of Ca^{2+} and myosin light chain phosphorylation in regulation of smooth muscle. *Am. J. Physiol.* 242:C109–16
55. Gerthoffer, W. T., Murphy, R. A. 1983. Myosin phosphorylation and regulation of the cross-bridge cycle in tracheal smooth muscle. *Am. J. Physiol.* 244:C182–87
56. Gerthoffer, W. T., Murphy, R. A. 1983. Ca^{2+}, myosin phosphorylation, and relaxation of arterial smooth muscle. *Am. J. Physiol.* 245:C271–77
57. Butler, T. M., Siegman, M. J., Mooers, S. U. 1983. Chemical energy usage during shortening and work production in mammalian smooth muscle. *Am. J. Physiol.* 244:C234–42
58. Silver, P., Stull, J. T. 1983. Effects of the calmodulin antagonist, fluphenazine, on phosphorylation of myosin and phosphorylase in intact smooth muscle. *Mol. Pharmacol.* 23:665–70
59. Kamm, K. E., Stull, J. T. 1985. Myosin phosphorylation, force and V_o in neurally stimulated tracheal smooth muscle. *Am. J. Physiol.* In press
60. de Lanerolle, P., Stull, J. T. 1980. Myosin phosphorylation during contraction and relaxation of tracheal smooth muscle. *J. Biol. Chem.* 255:9993–10000
61. de Lanerolle, P., Nishikawa, M., Yost, D. A., Adelstein, R. S. 1984. Increased phosphorylation of myosin light chain kinase after an increase in cyclic AMP in intact smooth muscle. *Science* 223:1415–17
62. Barron, J. R., Bárány, M., Bárány, K., Storti, R. V. 1980. Reversible phosphorylation and dephosphorylation of the 20,000 dalton light chain of myosin during the contraction-relaxation-contraction cycle of arterial smooth muscle. *J. Biol. Chem.* 255:6238–44
63. Janis, R. A., Bárány, K., Bárány, M., Sarmiento, G. 1981. Association between myosin light chain phosphorylation and contraction of rat uterine smooth muscle. *Mol. Pharmacol.* 1:3–11
64. Nishikori, K., Weisbrodt, N. W., Sherwood, O. D., Sanborn, B. M. 1983. Effects of relaxin on rat uterine myosin light chain kinase activity and myosin light chain phosphorylation. *J. Biol. Chem.* 258:2468–74
65. Silver, P. J., Stull, J. T. 1982. Regula-

tion of myosin light chain and phosphorylase phosphorylation in tracheal smooth muscle. *J. Biol. Chem.* 257:6145–50
66. Somlyo, A. V., Butler, T. M., Bond, M., Somlyo, A. P. 1981. Myosin filaments have nonphosphorylated light chains in relaxed smooth muscle. *Nature* 294:567–69
67. Aksoy, M. O., Mras, S., Kamm, K. E., Murphy, R. A. 1983. Ca^{++}, cAMP, and changes in myosin phosphorylation during contraction of smooth muscle. *Am. J. Physiol.* 245:C255–70
68. Haeberle, J. R., Hott, J. W., Hathaway, D. R. 1984. Pseudophosphorylation of the smooth muscle 20,000 dalton myosin light chain: An artifact due to protein modification. *Biochim. Biophys. Acta.* 790:78–86
69. de Lanerolle, P., Condit, J. R. Jr., Tanenbaum, M., Adelstein, R. S. 1982. Myosin phosphorylation, agonist concentration and contraction of tracheal smooth muscle. *Nature* 298:871–72
70. Ledvora, R. F., Bárány, K., Van der Meulen, D. L., Barron, J. T., Bárány, M., 1983. Stretch-induced phosphorylation of the 20,000-dalton light chain of myosin in arterial smooth muscle. *J. Biol. Chem.* 258:14080–83
71. Silver, P. J., Stull, J. T. 1982. Quantitation of myosin light chain phosphorylation in small tissue samples. *J. Biol. Chem.* 257:6137–44
72. Bárány, K., Sayers, S. T., DiSalvo, J., Bárány, M. 1983. Two-dimensional electrophoretic analysis of myosin light chain phosphorylation in the heart. *Electrophoresis* 4:138–42
73. Silver, P. J., Stull, J. T. 1984. Differential regulation of myosin light chain and phosphorylase phosphorylation by K^+ depolarization and carbamylcholine in tracheal smooth muscle. *Mol. Pharmacol.* 25:267–74
74. Dillon, P. F., Aksoy, M. O., Driska, S. P., Murphy, R. A. 1981. Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science* 211: 495–97
75. Morgan, J. P., Morgan, K. G. 1982. Vascular smooth muscle: The first recorded Ca^{2+} transients. *Pflügers Arch.* 395:75–77
76. Murphy, R. A., Aksoy, M. O., Dillon, P. F., Gerthoffer, W. T., Kamm, K. E. 1983. The role of myosin light chain phosphorylation in regulation of the cross-bridge cycle. *Fed. Proc.* 42:51–56
77. Bolton, T. B. 1979. Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.* 59: 606–718
78. Arner, A. 1982. Mechanical characteristics of chemically skinned guinea-pig taenia coli. *Pflügers Arch.* 395:277–84
79. Arner, A. 1983. Force-velocity relation in chemically skinned rat portal vein: Effects of Ca^{2+} and Mg^{2+}. *Pflügers Arch.* 397:6–12
80. Paul, R. J., Doerman, G., Zeugner, C., Rüegg, J. C. 1983. The dependence of unloaded shortening velocity on Ca^{++}, calmodulin, and duration of contraction in "chemically skinned" smooth muscle. *Circ. Res.* 53:342–51
80a. Siegman, M. J., Butler, T. M., Mooers, S. U., Michalek, A. 1984. Ca^{2+} can affect V_{max} without changes in myosin light chain phosphorylation in smooth muscle. *Pflügers Arch.* 401:385–90
81. Chatterjee, M., Murphy, R. A. 1983. Calcium-dependent stress maintenance without myosin phosphorylation in skinned smooth muscle. *Science* 221:464–66
82. Siegman, M. J., Butler, T. M., Mooers, S. U., Davies, R. E. 1976. Crossbridge attachment, resistance to stretch, and viscoelasticity in resting mammalian smooth muscle. *Science* 191:383–85
83. Meiss, R. A. 1982. Transient responses and continuous behavior of active smooth muscle during controlled stretches. *Am. J. Physiol.* 242:C146–58
84. Gerthoffer, W. T., Trevethick, M. A., Murphy, R. A. 1984. Myosin phosphorylation and cyclic adenosine 3',5'-monophosphate in relaxation of arterial smooth muscle by vasodilators. *Circ. Res.* 54:83–89
85. Arner, A., Hellstrand, P. 1983. Activation of contraction and ATPase activity in intact and chemically skinned smooth muscle of rat portal vein: Dependence on Ca^{++} and muscle length. *Circ. Res.* 53:695–702
86. Krisanda, J. M., Paul, R. J. 1984. Energetics of isometric contraction in porcine carotid artery. *Am. J. Physiol.* 246:C510–79
87. Stull, J. T., Blumenthal, D. K., Miller, J. R., DiSalvo, J. 1982. Regulation of myosin phosphorylation. *J. Mol. Cell Cardiol.* 14:105–10
88. Kramer, G. L., Hardman, J. G. 1980. Cyclic nucleotides and blood vessel contraction. See Ref. 5, pp. 179–99
89. Kuriyama, H., Ito, Y., Suzuki, H., Kitamura, K., Itoh, T. 1982. Factors modifying contraction-relaxation cycle in vascular smooth muscle. *Am. J. Physiol.* 243:H641–62
90. Krall, J. F., Fortier, M., Korenman, S.

G. 1983. Smooth muscle cyclic nucleotide biochemistry. See Ref. 8, 3:89–128

91. Kroeger, E. A. 1983. Roles of cyclic nucleotides in modulating smooth muscle function. See Ref. 8, 3:129–39
92. Casteels, R., Raeymaekers, L. 1979. The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.* 294:51–68
93. Mueller, E., van Breeman, C. 1979. Role of intracellular Ca^{2+} sequestration in β-adrenergic relaxation of a smooth muscle. *Nature* 281:682–83
94. Van Eldere, J., Raeymaekers, L., Casteels, R. 1982. Effect of isoprenaline on intracellular Ca uptake and on Ca influx in arterial smooth muscle. *Pflügers Arch.* 395:81–83
95. Itoh, T., Izumi, H., Kuriyama, H. 1982. Mechanisms of relaxation induced by activation of β-adrenoreceptors in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.* 326:475–93
96. Meisheri, K. D., van Breemen, C. 1982. Effects of β-adrenergic stimulation on calcium movements in rabbit aortic smooth muscle: Relationship with cyclic AMP. *J. Physiol.* 331:429–41
97. Bulbring, E., den Hertog, A. 1980. The action of isoprenaline on the smooth muscle of the guinea-pig taenia coli. *J. Physiol.* 305:277–96
98. Scheid, C. R., Fay, F. S. 1984. β-Adrenergic effects on transmembrane ^{45}Ca fluxes in isolated smooth muscle cells. *Am. J. Physiol.* 246:C431–38
99. Scheid, C. R., Honeyman, T. W., Fay, F. S. 1979. Mechanism of β-adrenergic relaxation of smooth muscle. *Nature* 277:32–36
100. Scheid, C. R., Fay, F. S. 1984. β-Adrenergic stimulation of ^{42}K influx in isolated smooth muscle cells. *Am. J. Physiol.* 246:C415–21
101. Adelstein, R. S., Conti, M. A., Hathaway, D. R. 1978. Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3':5' - monophosphate - dependent protein kinase. *J. Biol. Chem.* 253:8347–50
102. Silver, P. J., DiSalvo, J. 1979. Adenosine 3':5'-monophosphate-mediated inhibition of myosin light chain phosphorylation in bovine aortic actomyosin. *J. Biol. Chem.* 254:9951–54
103. Conti, M. A., Adelstein, R. S. 1981. The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J. Biol. Chem.* 256:3178–81
104. Vallet, B., Molla, A., Demaille, J. G. 1981. Cyclic adenosine 3',5'-monophosphate-dependent regulation of purified bovine aortic calcium/calmodulin-dependent myosin light chain kinase. *Biochim. Biophys. Acta* 674:256–64
105. Bhalla, R. C., Sharma, R. V., Gupta, R. C. 1982. Isolation of two myosin light-chain kinases from bovine carotid artery and their regulation by phosphorylation mediated by cyclic AMP-dependent protein kinase. *Biochem. J.* 203:583–92
106. Wolf, H., Hofmann, F. 1980. Purification of myosin light chain kinase from bovine cardiac muscle. *Proc. Natl. Acad. Sci. USA* 77:5852–55
107. Edelman, A. M., Krebs, E. G. 1982. Phosphorylation of skeletal muscle myosin light chain kinase by the catalytic subunit of cAMP-dependent protein kinase. *FEBS Lett.* 138:293–98
108. Kerrick, W. G. L., Hoar, P. E. 1981. Inhibition of smooth muscle tension by cyclic AMP-dependent protein kinase. *Nature* 292:253–55
109. Sparrow, M. P., Pfitzer, G., Gagelmann, M., Rüegg, J. C. 1984. Effect of calmodulin, Ca^{2+}, and cAMP protein kinase on skinned tracheal smooth muscle. *Am. J. Physiol.* 246:C308–14
110. Nishikawa, M., de Lanerolle, P., Lincoln, T. M., Adelstein, R. S. 1984. Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* 259:8429–36
111. Yeaman, S. J., Cohen, P., Watson, D. C., Dixon, G. H. 1977. The substrate specificity of adenosine 3':5'-cyclic monophosphate-dependent protein kinase of rabbit skeletal muscle. *Biochem. J.* 162:411–21
112. Pilkis, S. J., El-Maghrabi, M. R., Coven, B., Claus, T. H., Tager, H. S., et al. 1980. Phosphorylation of rat hepatic fructose-1,6-bisphosphatase and pyruvate kinase. *J. Biol. Chem.* 255:2770–75
113. Proud, C. G., Rylatt, D. B., Yeaman, S. J., Cohen, P. 1977. Amino acid sequences at the two sites on glycogen synthetase phosphorylated by cyclic AMP-dependent protein kinase and their dephosphorylation by protein phosphatase-III. *FEBS Lett.* 80:435–42
114. Rüegg, J. C., Sparrow, M. P., Mrwa, U. 1981. Cyclic-AMP mediated relaxation of chemically skinned fibres of smooth muscle. *Pflügers Arch.* 390:198–201

115. Rüegg, J. C., Paul, R. J. 1982. Vascular smooth muscle. Calmodulin and cyclic AMP-dependent protein kinase alter calcium sensitivity in porcine carotid skinned fibers. *Circ. Res.* 50:394–99
116. Meisheri, K. D., Rüegg, J. C. 1983. Dependence of cyclic-AMP induced relaxation on Ca^{2+} and calmodulin in skinned smooth muscle of guinea pig *Taenia coli*. *Pflügers Arch.* 399:315–20
117. Jones, A. W., Bylund, D. B., Forte, L. R. 1984. cAMP-dependent reduction in membrane fluxes during relaxation of arterial smooth muscle. *Am. J. Physiol.* 246:H306–11