Biochemical Basis for Contraction of Vascular Smooth Muscle*

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Some of the current facts and theories concerning the contractile mechanism in smooth muscle are summarized. The review is divided into two major sections. One deals with the components of the contractile apparatus (the thick and thin filaments), and the protein components of each filament type are described briefly. The other is devoted to the Ca\^{2+}-dependent mechanism of regulation in smooth muscle, and this is restricted to those components that control the activity of the contractile apparatus. There are basically two theories that have been proposed as the regulatory mechanism in smooth muscle. One theory is that the regulatory mechanism is located on the thin filaments and is functional via some modification of the thin filament proteins. This system is termed leiotonin. The second theory is that regulation is achieved by the phosphorylation and dephosphorylation of the light chains of myosin. Since the latter theory represents the author's bias and in general is more widely accepted, it is considered in more detail. A cyclic scheme is presented to illustrate the role of the myosin light chain kinase in the activation and contraction of smooth muscle and the role of the myosin light chain phosphatase in the relaxation process.

It is generally accepted that fluctuations of the free Ca\^{2+} level within a cell regulates several cellular processes. A regulatory function for Ca\^{2+} is certainly true for all types of muscle tissue where an increase or decrease of the Ca\^{2+} concentration results in contraction or relaxation, respectively. The intracellular Ca\^{2+} concentration is controlled through one or more membrane-associated Ca\^{2+} pumps (for example, the sarcoplasmic reticulum) and a consideration of this process is beyond the scope of this review. My main concern is to address the problem of what recognizes the Ca\^{2+} fluctuations and translates these to result in either contraction and relaxation. To illustrate this point, one can consider the situation in skeletal and cardiac muscle where it is known that troponin, which is located on the thin filament, is the target for the activating Ca\^{2+}. The regulation of contractile activity is then dictated by specific Ca\^{2+}-dependent interactions between the three troponin subunits, tropomyosin and actin.1 The consensus of opinion is that a troponin-like mechanism does not operate in smooth muscle and a different regulatory process is involved. What the current hypotheses are will be discussed below. The review is not intended to be comprehensive, and a more extensive treatment can be found elsewhere.*

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CONTRACTILE APPARATUS

Sliding Filament Mechanism

A fundamental component of any treatment of the contractile process in skeletal muscle involves a consideration of the sliding filament mechanism. It was established over 20 years ago that the length changes of striated muscle occur as a result of interdigitation of thick and thin filaments, where neither filament itself altered its length. Subsequently, it was shown that the thick filament is composed predominantly of myosin (minor protein constituents are present, for example, the C-protein) and the thin filament is composed of three proteins. The backbone of the thin filament is a double strand of helically-wound actin, F-actin, and superimposed on this are the regulatory proteins, troponin and tropomyosin. At the time that the double filament array was discovered in skeletal muscle and, indeed for the following several years, a similar situation could not be demonstrated in smooth muscle. Thin filaments were numerous, but the earlier attempts to visualize the thick filaments were unsuccessful. It was subsequently discovered that thick filaments were indeed present in smooth muscle, but were more labile than their striated muscle counterparts. As the fixation techniques improved, the demonstration of thick filaments in many types of smooth muscle became commonplace and it is now accepted that the thick and thin filament array exists in...
smooth muscle. This is not merely the resolution of a technical controversy, but it is important to the basic understanding of smooth muscle biochemistry since it opens up the possibility that a sliding filament mechanism might account for the length changes in smooth as well as in skeletal muscle. Most investigators, in fact, assume this to be the case, and all of the evidence is consistent with a sliding filament mechanism in smooth muscle.

The sliding filament concept is based on the interaction of the myosin cross-bridges with the actin filaments. The cross-bridge is formed from an extension of the myosin molecule which protrudes from the body of the thick filament and which contains at the tip of the cross-bridge, the ATPase hydrolysis sites and actin binding sites. In relaxed striated muscle it is known that the cross-bridges do not bind to actin and the two filament types can slide freely past each other. In contracting muscle, however, the cross-bridge actin interaction is the site for ATP hydrolysis and tension development. Thus, one fundamental distinction between the states of contraction and relaxation is in the interaction of the cross-bridges with actin. It follows that the mechanism which brings about relaxation must, directly or indirectly, reduce the number of cross-bridge contacts.

Since the cross-bridge is the site for force development, it is reasonable to assume that the tension developed is proportional to the number of cross-bridges which are operating. This has been established for skeletal muscle where it was found that tension development is related to the number of cross-bridges acting in parallel. This in turn must be related in some way to the content of myosin in a given muscle. The logical situation would be that a greater number of potential cross-bridges would be present in muscles containing more myosin. It is interesting in this respect that, in general, smooth muscles contain less myosin than skeletal muscle and yet can develop similar tensions. For example, the myosin content of a number of smooth muscles was estimated to be about 20 mg per g cell wet weight compared to about 62 mg per g for skeletal muscle. The explanation behind this apparently anomalous situation is not established, although several suggestions have been made. These include: longer filaments, both thick and thin, in smooth muscle; different thick filament packing mode; and, a longer contact time of the cross-bridge with actin during the cross-bridge cycle. Some of these possibilities are considered in more detail by Murphy.

Another feature of the protein composition that differs between skeletal and smooth muscle is in the relative actin contents. Arterial smooth muscle contains about 50 mg/g cell weight and non-arterial smooth muscle (ie esophagus, trachea, intestine) about 25 mg/g cell weight, compared to 22 mg/g cell weight for skeletal muscle. The differences are more dramatically expressed as weight ratios of actin:myosin, which are approximately 2.6, 1.5 and 0.36 for arterial smooth muscle, non-arterial smooth muscle and skeletal muscle, respectively. These translate to molar actin:myosin ratios of about 29, 17 and 4, respectively. The higher actin content is reflected by a corresponding increase in tropomyosin, which is bound to the actin filament, although contrary to earlier ideas, the molar stoichiometry of actin to tropomyosin is 6 to 7:1, which is similar to the value obtained with skeletal muscle.

In terms of the structure and composition of the two filament types in smooth and skeletal muscle, there are some differences. The thick filaments in smooth muscle are probably longer by about 40 percent than their skeletal counterparts and the manner in which the myosin molecules are arranged to form the filament could also be different. A distinct clear central zone, which is characteristic of the bipolar packing of the molecules in the skeletal filament, is not obvious in the filaments from smooth muscle. This might indicate that the myosin molecules are packed so that their heads are oriented only in one direction, ie, where each side or face of the filament has the same polarity. The major difference with respect to the thin filaments is in their protein composition. As stated above the skeletal thin filament is composed of actin, tropomyosin and troponin, whereas in the thin filament from smooth muscle troponin is absent. In gross appearance, as seen by the electron microscope, the thin filaments from the two muscle types are indistinguishable.

**Major Protein Components**

The proteins of the thick and thin filaments from each muscle type are basically the same, although some differences are apparent in subunit compositions. For example, myosin from all muscle types contains two subunits of about 200,000 daltons each, and two pairs of smaller subunits, called light chains. The light chain composition, to a degree, characteristic of a particular myosin. Myosin from smooth muscle contains two light chains of 20,000 daltons, and two light chains of 17,000 daltons; myosin from cardiac muscle has two classes of light chains of 27,000 and 20,000 daltons, and myosin from white skeletal muscle has two 18,000 dalton light chains and two subunits of either 16,000 or 25,000 daltons. In general, the myosin from nonmuscle sources resembles the smooth muscle type in its light chain composition. It is known that the removal of the
light chains from the two heavier subunits (by the use of extreme pH, or urea or guanidine) results in the loss of ATPase activity. In smooth muscle (and probably nonmuscle myosins) it has been suggested (see section on Regulation) that the phosphorylation of the 20,000 light chain allows the activation by actin of the Mg\(^{2+}\)-ATPase activity of the myosin, and that this phosphorylation is an essential component of the regulatory mechanism. The light chains of many invertebrate myosins\(^{6,7}\) are also involved in the Ca\(^{2+}\)-control process where it has been demonstrated that the binding of Ca\(^{2+}\) to the myosin light chains is necessary for the activation of ATPase activity. In the case of vertebrate striated myosins, the function of the light chains is not obvious and it is apparent that modification of the light chains, either by ion binding or phosphorylation, does not elicit dramatic changes in the myosin ATPase activities.

The major proteins of the smooth muscle thin filament, actin and tropomyosin, also show some differences when compared to the skeletal muscle proteins. But these distinctions are relatively slight, especially in the case of actin.\(^{\ast}\) With respect to their functional properties the proteins of the thin filaments are very similar. For example, tropomyosin from chicken gizzard has an apparent difference in its subunit composition (as judged by sodium dodecyl sulfate polyacrylamide electrophoresis) when compared to tropomyosins from striated muscles.\(^{6}\) However, it exhibits all of the relevant properties that characterize the skeletal tropomyosin molecule; it binds to actin with a similar stoichiometry, 1 tropomyosin to 7 actins; it forms end-to-end polymers at low ionic strength; it forms a complex with skeletal troponin and can regulate the activity of skeletal actomyosin. Thus, while tropomyosin does not appear to be an essential component of the regulatory mechanism in smooth muscle the molecule still possesses all of the properties that allow tropomyosin to fulfill a regulatory function in skeletal muscle. In a similar fashion, skeletal and smooth muscle actins are largely interchangeable with respect to their functional properties.

Concluding Comments

It is generally assumed that the basic contractile mechanisms in skeletal and smooth muscles are similar, and involve the relative sliding, or interdigitation, of thick and thin filaments.\(^{\dagger}\) The focus of tension development is the myosin cross-bridge, which undergoes repeated cross-bridge cycles with the concomitant hydrolysis of one ATP molecule per cycle. There is very little known about the kinetics of the cross-bridge cycle in smooth muscle, although it is assumed that the rate limiting step in the cycle is the release of ADP and P\(_i\) (as it is in skeletal muscle). The most striking difference between the skeletal and smooth systems is the rate of the cross-bridge cycles. It has been calculated\(^{9}\) for arterial muscle at 37\(^\circ\)C that the cycling rate is of the order of 1 sec\(^{-1}\), which is at least an order of magnitude slower than most skeletal muscles. Since the cross-bridge is composed of the enzymatically active portion of the myosin molecule, it is to be expected that the low cycling rate is reflected by a low specific ATPase activity of myosin. To a large extent, this is justified as the Mg\(^{2+}\)-activated ATPase activity of actomyosin from smooth muscle is in the range of 20 to 200 n moles phosphate liberated min\(^{-1}\)mg\(^{-1}\) myosin, giving turnover numbers of 0.15 to 1.5 sec\(^{-1}\). If one assumes a Q\(_{10}\) of close to 3, values of about 0.54 to 5.4 sec\(^{-1}\) would be obtained for 37\(^\circ\)C. Another feature that is often quoted as characteristic of smooth muscle is the low ATP usage for tension maintenance. This "catch-like" property is probably partly due to the slower cross-bridge cycle, although it remains to be established whether or not this explanation can fully account for the high economy of tension maintenance in smooth muscle.

A final point to be emphasized is that tension is generated only as a consequence of the interaction of the myosin cross-bridge with actin, and this corresponds to the actin-activation of the myosin ATPase activity. In relaxed muscle, contacts with actin are not made and the two filament types are separated. Thus, the regulatory mechanism must operate by controlling the contacts of the cross-bridges with actin. The biochemical "model" for this is to measure the activation and inhibition of the Mg\(^{2+}\)-ATPase activity of actomyosin which would correspond to contraction and relaxation, respectively.

Regulatory Mechanism

Historic

Often in the area of smooth muscle biochemistry, the state of the art at any given time follows, and to some extent depends upon, advances made in corresponding areas of skeletal muscle research. The initial investigations into the regulatory mechanism of smooth muscle were no exception to this generalization and were stimulated by the discovery of troponin in skeletal muscle by Ebashi and colleagues. Indeed, there are a few reports which claimed...
that smooth muscle also was regulated by a troponin-like mechanism, although these were not confirmed. A second milestone came from the work of Szent-Györgyi and colleagues, who found that molluscan muscle was regulated by the binding of Ca\(^{2+}\) to two of the myosin light chains. The regulatory mechanism was therefore a property of the myosin molecule (myosin-linked) and was not dependent on the interactions of the thin filament proteins as in vertebrate skeletal and cardiac muscle. Further, a test was devised which distinguished between the myosin-linked and the actin-linked types of regulation. When this test was applied to a smooth muscle system it was found by Bremel that the regulatory system was centered on the myosin molecule. This was a critical observation as it focused the subsequent search for the regulatory proteins to factors which modified myosin rather than following the "classic" thin filament model. The situation, however, was not as simple as that in the invertebrate scheme. The difficulty that was encountered was that as the smooth muscle myosin was purified, its actin-activated ATPase activity became progressively less until it was negligible. It was obvious, therefore, that in order to achieve a Ca\(^{2+}\)-dependent regulation, an additional factor(s) was required which activated the Mg\(^{2+}\)-ATPase activity of actomyosin and whose influence was directed towards the myosin molecule. The system, therefore, would still be "myosin-linked," but would be more complex than the simplest molluscan system.

**Requirement for the Regulatory System**

The basic requirement for the regulatory mechanism in smooth muscle is that the Mg\(^{2+}\)-ATPase activity of actomyosin is activated in the presence of Ca\(^{2+}\) at those Ca\(^{2+}\) concentrations necessary to initiate contraction, and that in the absence of Ca\(^{2+}\) no activation is achieved. This type of control mechanism is also thought to be operative in non-muscle-systems. It should be emphasized that the fundamental concept for this mechanism is quite different from that which occurs in skeletal and cardiac muscles. This can be illustrated by the following experiment: if one prepares pure myosin and pure actin from skeletal and cardiac sources, then the Mg\(^{2+}\)-ATPase activity of the resultant actomyosin is close to maximal. The ATPase does not show any Ca\(^{2+}\)-dependence (ie it is unregulated) but it is active. This is not the case if the same experiment is performed using smooth muscle myosin and actin. Thus, the function of the regulatory proteins in striated muscle (troponin and tropomyosin) is to inhibit the ATPase activity of actomyosin, but only in the absence of Ca\(^{2+}\), and the function of the regulatory proteins in smooth muscle is to activate the Mg\(^{2+}\)-ATPase activity of actomyosin, but only in the presence of Ca\(^{2+}\).

That an activator is required in the smooth muscle system is generally acknowledged, the controversy that exists concerns the nature of that activator. Three theories can be considered: 1) that a troponin-like system is operative; 2) that activation is achieved as a result of the phosphorylation of the 20,000 dalton light chains of myosin; and 3) that a system, termed leiotonin, is the activating principle. The first suggestion, however, is not a very plausible possibility. It is now realized that the troponin-C like protein which was isolated from several smooth muscles, is probably calmodulin, which is found in almost all eucaryotic cells. The existence of the other troponin subunits, troponin I and troponin T, has not been established. There is also the consideration that the mode of action of troponin in striated muscle is to inhibit an active actomyosin complex. Therefore, if troponin does exist in smooth muscle, it must either be different than its striated counterpart, or if similar, it must operate as a secondary control mechanism to moderate the actomyosin complex which is activated by another system. Since at this time there is very little evidence to support the "troponin-theory" only the phosphorylation and leiotonin mechanisms will be considered below.

**Leiotonin**

This system was discovered by Ebashi and colleagues and was suggested to be the regulatory component of smooth muscle. It is similar to the phosphorylation mechanism in that it causes an activation of the Mg\(^{2+}\)-ATPase activity of actomyosin in the presence of Ca\(^{2+}\), but it differs in many other respects. The major distinction between the leiotonin and the phosphorylation theories is that the activation of actomyosin ATPase activity, or super-precipitation (an "in-vitro" analog of contraction) is thought to be achieved by leiotonin without the phosphorylation of the myosin molecule. The system is thought to be located on the thin filaments where it is functional at relatively high actin:leiotonin ratios, of about 100:1. Evidence in support of the actin-linked nature of leiotonin has recently been obtained by Mikawa who was able to "freeze" the thin filaments of smooth muscle in either an active, or "on" state, and an inactive, or "off" state, by the use of cross-linking with gluteraldehyde. This would argue in favor of a control system which modified the co-factor, actin, rather than altering directly the myosin molecule. Leiotonin also shows a preference for smooth muscle actin and tropomyosin which is not evident with the phosphorylation system.
recently, leiotonin has been separated into two components named leiotonin A and C. The molecular weights of the subunits are about 80,000 and 18,000, respectively. The latter is an acidic protein which is similar to, but not identical with, calmodulin.

The mechanism of action of leiotonin is not known, and the elucidation of this must precede the evaluation of the two potential control processes. It is thought that since leiotonin is functional at such high actin:leiotonin ratios, its role could not be structural (as is for example, that of troponin) and this could indicate a catalytic function for leiotonin. It is possible, of course, that two independent regulatory systems exist in smooth muscle, but however comforting this might be, it is at this stage pure conjecture.

**Phosphorylation as a Regulatory Mechanism**

This theory offers the most widely accepted explanation for the control process in smooth muscle. It was discovered by Sobieszek and reported in 1975. Since that time, many independent groups have confirmed and extended the original observations. The basic facts that constitute the theory are that: 1) the two 20,000 dalton light chains of the myosin molecule can be phosphorylated (1 mole of phosphate per mole of light chain) by a specific enzyme, the myosin light chain kinase (MLCK); 2) phosphorylation of the light chains occurs only in the presence of Ca\(^{2+}\) and at Ca\(^{2+}\) concentrations the same as those required to initiate contraction of smooth muscle; 3) the event of phosphorylation allows the activation by actin of the Mg\(^{2+}\)-ATPase activity of myosin, thus fulfilling the requirement for an activator; and 4) an additional enzyme exists, a myosin light chain phosphatase, which removes the phosphate groups from the myosin light chains and returns the actomyosin to its dormant state.

There is considerable experimental evidence to support each of the above statements and if one accepts their validity then a tentative scheme to illustrate the role of phosphorylation of myosin as a regulatory mechanism in smooth muscle can be proposed as in Figure 1. Myosin is phosphorylated by the MLCK in the presence of Ca\(^{2+}\). This event can be regarded as an activation step preliminary and essential to the onset of contraction. In the presence of actin, the phosphorylated myosin forms actomyosin which will undergo repetitive cycles of ATP hydrolysis (corresponding to the cross-bridge cycles) and this will continue as long as Ca\(^{2+}\) is present. This phase can be considered as that occurring during steady-state tension development. When Ca\(^{2+}\) is removed, either by sequestration within a sarcoplasmic reticulum system or by active transport through the cell membrane, the MLCK becomes inactive and the phosphatase removes the phosphate groups from the myosin light chains. This results in the dissociation of the actomyosin and the relaxation of the muscle.

There are several aspects of this scheme which will be considered in more detail and for convenience these have been itemized (1 through 5) in Figure 1.

1) **The nature of the myosin light chain kinase (MLCK).** One of the initial objectives in our studies

![Diagram](http://journal.publications.chestnet.org/pdfaccess.ashx?url=data/journals/chest/21159/)
on the regulatory mechanism in smooth muscle was to purify and characterize the MLCK. During the course of these experiments it was found that when the crude kinase was fractionated on Sepharose 4-B, none of the eluted components possessed any kinase activity when assayed individually. Activity was recovered only when one of the larger components was combined with one of the smaller components. The two components were purified and it was concluded that the MLCK was composed of two subunits of molecular weights, 105,000 and 17,000.\(^{15}\) Neither component alone possessed any kinase activity. The smaller subunit was identified subsequently\(^ {16}\) as calmodulin (other terms for this protein include, phosphodiesterase activator protein, modulator protein and calcium dependent regulator protein) which was known to be involved in the regulation of cyclic nucleotide metabolism.\(^ {17}\) Largely by analogy with the role of calmodulin in the activation of phosphodiesterase\(^ {17}\) the following sequence of events can be proposed for the activation of the MLCK: in the relaxed smooth muscle cell the calmodulin is thought to exist as an isolated component, i.e., not in association with other proteins. On excitation of the muscle, the level of intracellular Ca\(^ {2+}\) increases and the Ca\(^ {2+}\)-calmodulin complex is formed (up to 4 moles Ca\(^ {2+}\)/mole protein). The Ca\(^ {2+}\)-calmodulin complex interacts with the 105,000 subunit (or with any of the other calmodulin-dependent systems) to form a ternary complex which is active and phosphorylates the myosin light chains. The stoichiometry of the active complex involves one molecule each of the two subunits.

At about the same time that our work on smooth muscle was done it was found by Yazawa and Yagi\(^ {18}\) that a MLCK from skeletal muscle also required calmodulin for its activity. Some differences in the skeletal and smooth muscle systems are apparent and it was found that the larger kinase subunit in skeletal muscle has a molecular weight of about 80,000. However, the most significant difference between the two muscle types is that the phosphorylation of skeletal muscle myosin does not result in any pronounced changes in its ATPase properties, and in fact the function of the MLCK in skeletal muscle is unknown. More recently, other systems have been examined and calmodulin-dependent MLCK has been detected in turkey gizzard\(^ {19}\) and in blood platelets.\(^ {20}\) In the former, the larger kinase subunit was reported to be about 125,000 molecular weight.

Thus, to summarize this section, it can be proposed that the active complex of the MLCK consists of two protein subunits plus Ca\(^ {2+}\). The calmodulin component provides the Ca\(^ {2+}\)-dependence for the system and is the receptor for the activating Ca\(^ {2+}\). The larger kinase subunit provides the active site for the phosphotransferase activity. The type of regulation exhibited by calmodulin-dependent processes is quite distinct from the well known regulation of the cAMP-dependent protein kinase, where the dissociated catalytic subunit is active and the complex with the regulatory subunit is inactive. With the MLCK (and probably also with the other calmodulin-associated systems) the situation is reversed and the complex is active and the dissociated components inactive.*

2) Activation phase: The assumption that is critical for the acceptance of the phosphorylation theory is that the phosphorylation of the myosin molecule is a prerequisite for the subsequent activation of the contractile apparatus. In a biochemical sense one could rephrase this to state that phosphorylation is essential for the activation of Mg\(^ {2+}\)-ATPase activity of actomyosin. Although this relationship has not received the exhaustive analysis that it deserves, it is certainly true that the experimental evidence that does exist shows a dependence of ATPase activity on the state of myosin phosphorylation. For example, in our laboratory, using the purified kinase system, it was shown that the increase of the specific Mg\(^ {2+}\)-ATPase activity of actomyosin paralleled the increase in the extent of myosin phosphorylation.\(^ {18}\) (The use of the purified kinase components also reduced the possibility that the activation was due to a contaminant in the kinase preparations.) Other in vitro evidence in favor of the phosphorylation theory include: the use of ATP-S, which will be discussed below; the correlation between the rate of dephosphorylation and the actomyosin ATPase activity;\(^ {21}\) and the evidence using the subfragments of myosin (obtained by proteolysis) where a relationship exists between the ATPase properties of the subfragments and the amount of 20,000 light chain that each retains. The similar Ca\(^ {2+}\)-dependence of the phosphorylation of myosin and of ATPase activation of actomyosin should be considered as circumstantial supporting evidence, as it is simple to envisage that the similarities could be coincidental.

If the phosphorylation of myosin is required before contraction can occur, then the onset of contraction could not occur faster than the rate of myosin phosphorylation, and it is obviously important to establish the latter. This has been measured in sev-

*This applies to the normal in vivo system, but it can be altered by subjecting the kinase to mild proteolysis. The degraded kinase loses both its requirement for calmodulin and also its Ca\(^ {2+}\)-dependence. The sensitivity of the kinase to proteolysis is of practical concern and it is particularly troublesome when dealing with tissues containing high levels of proteolytic enzymes, e.g., uterus.
eral laboratories for MLCKs isolated from different sources. The values obtained at 25°C for some of the phosphorylation rates† (each expressed as μmoles P transferred min⁻¹mg⁻¹ kinase) are: 5-13 (chicken gizzard), approx 5 (turkey gizzard),¹⁹ 4-30 (skeletal muscle),¹⁸,²² and approx 3 (human blood platelets).²⁰ If one takes a value of 10 μmoles min⁻¹mg⁻¹ kinase as representative of smooth muscle this gives a turnover number of about 18 sec⁻¹. (The Q₁₀ for the kinase reaction is close to 2 so that the turnover number at 37°C would be about 43 sec⁻¹.) This value represents the rate at which the contractile apparatus can be activated. The steady-state ATPase activity of actomyosin, which is indicative of the cross-bridge cycling rate, is quite variable and values between 20 and 200 n moles phosphate liberated min⁻¹mg⁻¹ myosin have been obtained (see later section). Taking a value of 100 n moles min⁻¹mg⁻¹ as an average figure this gives a turnover number for the hydrolysis of ATP by actomyosin of about 0.8 sec⁻¹. (Mrwa et al²¹ calculated a cross-bridge turnover rate of 1 sec⁻¹ for arterial muscle at 37°C.) This means that the contraction speed as indicated by the specific activity of the actomyosin is at least an order of magnitude slower than the activation process which is given by the kinase rate. Thus, it would be expected that the phosphorylation of myosin is not a rate-limiting step in the contraction process. This assumes, of course, that the kinase is optimally active and this may not always be the case. Adelstein and co-workers²¹ have found recently that the cAMP-dependent protein kinase phosphorylates the larger subunit of the MLCK and that this results in an inhibition of the MLCK activity. Thus, it is possible that cAMP also plays a regulatory role in the actual contraction process in smooth muscle, and this interesting possibility must be explored in the future.

Other factors which have been tested for their effect on the phosphorylation rate should also be mentioned. It was found in our laboratory that one of the products of ATP hydrolysis, i.e., ADP, caused an inhibition of the kinase activity (this was found also for the skeletal muscle kinase by Perry and colleagues).²² However, this effect was variable and must be re-examined before any physiologic role for the ADP inhibition can be claimed. Factors which do not affect the kinase activity include: phosphate, pyrophosphate, IDP, AMP, adenosine, cAMP (in the absence of protein kinase), cGMP (all at concentrations not less than 100 μM). Finally, the presence of actin and/or tropomyosin does not alter the rate of phosphorylation of myosin.

3) Actomyosin ATPase activity: Once the myosin is phosphorylated, its Mg²⁺-ATPase activity is activated by actin, and this is the stage that is thought to reflect the contraction speed or the tension development of the muscle. As mentioned above, the reported values for the specific activity of actomyosin are variable and range between 20 to 200 n moles min⁻¹mg⁻¹ myosin, and it is therefore difficult to assign to a representative value. Part of the problem is due to the non-linear ATPase kinetics usually seen with smooth muscle actomyosin. This shows a rapid initial phase (about 100-200 n moles min⁻¹mg⁻¹ myosin) followed by a slower steady-state rate (in the order of 20 to 80 n moles min⁻¹mg⁻¹ myosin). Whether or not the biphasic response is representative of the physiologic situation is an interesting possibility, but this has not been established experimentally. One might speculate that the faster initial phase is reflective of a phasic response and that the slower second phase is characteristic of a more tonic contracture.

Why the phosphorylation of the myosin light chains alters the ATPase properties of the myosin active sites, which are located on the heavy chains, is not known, but some possibilities have been raised. One is that ATP (at mM concentrations) is inhibitory to unphosphorylated myosin but that the phosphorylation of the light chains removes the ATP inhibition. Another suggestion²⁰ is that the state of aggregation of the myosin molecules is related to ATPase activity, with the filamentous form showing higher activity. The effect of phosphorylation is thought to prevent the filaments from disassembling in the presence of ATP, and thus preserve a higher ATPase activity. The problem with this theory is that it requires the absence of thick filaments in relaxed muscle and their assembly during the activation phase. While this possibility has not been completely eliminated most of the available evidence indicates that thick filaments are present in both the relaxed and contracted states.

Another point which should be mentioned briefly in this section is the effect of tropomyosin. For the phosphorylation theory of regulation it is assumed by most investigators that tropomyosin does not play a direct or essential role (as opposed to its role in the regulation of skeletal muscle). However, tropomyosin from both skeletal and smooth muscles does stimulate the Mg²⁺-ATPase activity of phosphorylated smooth muscle actomyosin. (The extent of activation is between 50 percent and 100 percent of the initial ATPase activity.) Although it is clear that

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†These rates were obtained using the isolated myosin light chains as a substrate. It is not clear whether or not identical rates would be obtained using myosin as a substrate. In some laboratories, myosin is as effective as light chains, in other laboratories the rate using myosin is slower. Thus, the values quoted here should be regarded as tentative until the myosin rates are established.
the tropomyosin-actin complex (approximately 1:7 molar stoichiometry, respectively) is a better co-factor for myosin than actin alone, the molecular basis for the activation is not known.

4) Myosin light chain phosphatase: This enzyme has not been isolated and characterized from smooth muscle sources, although this has been achieved for the skeletal muscle phosphatase. However, the presence of the phosphatase has been detected in several smooth muscles and some of its properties are known. For instance the activity of the phosphatase is not influenced by Ca$^{2+}$ (or calmodulin) and the rate of dephosphorylation of phosphorylated light chains is relatively slow when compared to the kinase rate. Thus, it is thought that the phosphatase is active at all times, but in contracting muscle the kinase activity swamps the phosphatase activity and it is only when the kinase is inactivated by the removal of Ca$^{2+}$ that the phosphatase can achieve a net dephosphorylation of the myosin. If the dephosphorylation of myosin is the critical event in determining whether or not the muscle relaxes, then the rate of muscle relaxation should coincide with the rate of myosin dephosphorylation and this relationship should be tested. It is not known if the activity of the phosphatase is regulated in the in vivo state, although to date an in vitro regulation has not been observed. The phosphatase is relatively specific and will not hydrolyze p-nitrophenylphosphate, or catalyze the conversion of phosphorylase a to b.

The combined action of the myosin light chain kinase and phosphatase could lead to the release of phosphate as shown below:

\[
\text{ATP} \rightarrow \text{ADP} \rightarrow \text{Myosin-P} \rightarrow \text{Myosin + P}_1
\]

kinase, Ca$^{2+}$ phosphatase

However, this “pseudo-ATPase” activity is relatively minor and in a normal actomyosin preparation would comprise less than 2 percent of the total ATPase activity.

5) Role of Ca$^{2+}$ in the relaxation phase: It has been suggested that Ca$^{2+}$ initiates contraction through its interaction with calmodulin and the subsequent activation of the MLCK. It is known also that the removal of Ca$^{2+}$ results in the relaxation of muscle with the accompanying dephosphorylation of myosin. The simplest mechanism to account for relaxation is that the removal of Ca$^{2+}$ inactivates the kinase and thereby allows the phosphatase to dephosphorylate myosin. In this situation, the dominant feature is whether or not the myosin is phosphorylated and the only function of Ca$^{2+}$ is in regulating the activity of the kinase. However, an alternate mechanism is possible. It has been demonstrated that Ca$^{2+}$ binds to both skeletal and smooth muscle myosins and it was suggested that the Ca$^{2+}$-myosin interaction formed an important component of the regulatory mechanism (as it does in many invertebrate systems). In the first theory, phosphorylated myosin would be active both in the absence and presence of Ca$^{2+}$, but in the latter theory phosphorylated myosin would be active only in the presence of Ca$^{2+}$.

To decide between the two possibilities is a fairly simple experimental concept, but in practice this was complicated by the finding that the myosin was generally contaminated by trace amounts of kinase and phosphatase. Thus, the approach that was taken was to design experiments in which the effect of contaminants might be reduced. An example is the use of adenosine 5'-O-(3-thiotriphosphate), ATPyS. This analog serves as a substrate for the MLCK but the resultant thio phosphorylated light chain is a poor substrate for the phosphatase. The net effect is that myosin becomes locked into the thio phosphorylated state and the influence of Ca$^{2+}$ can be assayed, independent from any alterations in the state of phosphorylation. It was found that as the extent of thio phosphorylation increased the Mg$^{2+}$-ATPase activity of the actomyosin in the absence of Ca$^{2+}$ also increased until it reached the plus Ca$^{2+}$ ATPase level. This suggested that phosphorylated myosin is active regardless of whether or not Ca$^{2+}$ is present, and it does not support the idea that an additional regulatory site is present on the myosin molecule. Recently, the effect of ATPyS was tested using mechanically disrupted smooth muscle fibers and it was found that thiophosphorylation resulted in a loss of relaxation in the absence of Ca$^{2+}$. The reason that the above experiments are described is that the ATPyS results constitute compelling evidence in favor of the phosphorylation theory. It is difficult to imagine that these results could be obtained in a nonphosphorylation dependent system.

Physiologic evidence in support of the phosphorylation theory: Most of the earlier experiments were done using isolated protein preparations, although more recently various types of muscle fiber preparation have been examined. Barron et al. using strips of arterial muscle, found an increase in the phosphorylation of the myosin light chains when tension was produced on stimulation by norepinephrine or KCl. Contraction of myometrial strips also induced a marked increase in the extent of myosin phosphorylation. Kerrick et al. using skinned fibers of rabbit ileum and pulmonary artery, showed that
the calmodulin antagonists, trifluoperazine and chlorpromazine, induced relaxation of the muscle and further that this was associated with the dephosphorylation of myosin. The use of ATP$\gamma$S, by Cassidy et al.\textsuperscript{28} on muscle strips also provides very strong support for the phosphorylation theory of regulation. Thus, the evidence in favor of the phosphorylation theory has been accumulated using a variety of experimental techniques. Although it is probably true that while one single piece of experimental evidence is not conclusive, the cumulative evidence in favor of this mechanism of regulation is convincing.

**Summary of the phosphorylation mechanism of regulation in smooth muscle:** The most popular theory to account for the regulation of smooth muscle activity is based on the phosphorylation and dephosphorylation of the myosin light chains. In the presence of Ca$^{2+}$, the myosin light chain kinase phosphorylates myosin (2 moles phosphate per mole myosin) and thus allows the subsequent actin-activation of the Mg$^{2+}$-ATPase activity of myosin. Under these conditions, the muscle contracts. When Ca$^{2+}$ is removed, the kinase is inactivated and a second enzyme, a myosin light chain phosphatase, removes the phosphate groups from the myosin light chains, the actin-myosin complex dissociates and the muscle relaxes. The kinase therefore acts to activate the contractile apparatus and the phosphatase serves to deactivate the process. It was found that the kinase is composed of two subunits of molecular weights, approximately 100,000 and 17,000. The smaller component was identified as the Ca$^{2+}$-binding protein, calmodulin, which is also involved in several other enzymic mechanisms, including cyclic nucleotide metabolism.

*These compounds bind to calmodulin at relatively low concentrations and inhibit the calmodulin-dependent processes, eg the MLCK.

**References**

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