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Phil. Trans. R. Soc. Lond. B 1983 **302**, 59-71
doi: 10.1098/rstb.1983.0038

Phil. Trans. R. Soc. Lond. B **302**, 59–71 (1983) [59]
Printed in Great Britain

Phosphorylation of the myofibrillar proteins and the regulation of contractile activity in muscle

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Evidence now exists for the phosphorylation of all the major proteins of the myofibril with the exception of troponin C. Although uncertainty exists in most cases about the role of phosphorylation of the myofibrillar proteins, there is substantial evidence that phosphorylation of serine 20 of rabbit cardiac troponin I leads to a lowering of the sensitivity of the actomyosin ATPase to Ca^{2+}. This process is of special importance in the physiological response of the heart to adrenalin. A well defined enzymic system involving a specific kinase and a phosphatase is present in most muscles for the phosphorylation and dephosphorylation of the P light chain (regulatory, L2 or DTNB light chain) of myosin. Myosin light-chain kinase is very active in fast skeletal muscles, and although it is unlikely that phosphorylation followed by dephosphorylation of the P light chain occurs fast enough to be synchronous with the contractile cycle, phosphorylation may have a modulatory role in this tissue. Both post-tetanic potentiation and the reduced actomyosin ATPase turnover rate observed in fast-twitch muscle as a consequence of sustained forceful contraction have been suggested by different investigators to be consequences of P light chain phosphorylation. Nevertheless, unequivocal evidence associating either of these effects with phosphorylation is not yet available. Kinase activity is also high in vertebrate smooth muscle and it has been suggested that phosphorylation of the P light chain is the process that activates the actomyosin ATPase in this tissue. Evidence from a number of studies indicates, however, that regulation of smooth muscle actomyosin ATPase may not be a simple phosphorylation–dephosphorylation process.

Introduction

Three aspects of the specialized biochemistry of muscle that involve phosphorylation can be identified. These are: (1) the excitation contraction coupling process involving Ca^{2+} at the sarcoplasmic reticulum and at the myofibril; (2) regulation of the transduction process at the myofibril; (3) filament formation. The effects, particularly those summarized in (1) and (2), are largely concerned with modulating the contractile response in striated muscle that is already activated rather than converting the system from the inactive to the active form. In this respect the role of phosphorylation is different from that in enzyme systems such as phosphorylase or the hormone-sensitive lipase, where the process is involved in activation. In vertebrate smooth muscle, however, phosphorylation of myosin appears to have a role similar to that observed in other enzyme systems known to be regulated by phosphorylation. Although the evidence is not completely unequivocal as to whether phosphorylation of the P light chain is the trigger for contraction, the weight of experimental evidence is that it leads to an increase in the actin-activated ATPase. In this discussion attention will be directed to the effects of phosphorylation of the myofibrillar proteins on the transduction process.

In vertebrate muscle evidence exists for the phosphorylation of all the major myofibrillar proteins with the exception of troponin C (table 1). Regulation of contractile activity can occur

by direct interaction of Ca^{2+} with either A or I filament systems, depending on the muscle type; thus phosphorylation can modify the response through either system. The actomyosin system is basically similar in all muscles but the differences in contractile response of the different muscle types are produced by special features of the systems that activate and modulate the transduction process. It is in this function that phosphorylation of the different myofibrillar proteins assumes a particular importance.

TABLE 1. PROTEINS OF THE MYOFIBRIL THAT CAN BE PHOSPHORYLATED

(Specific kinases have been isolated for those proteins marked with an asterisk.)

A filament	*I filament*
myosin: P light chain*	troponin I
myosin: heavy chain	troponin T*
C protein	tropomyosin
	actin

PHOSPHORYLATION OF I FILAMENT

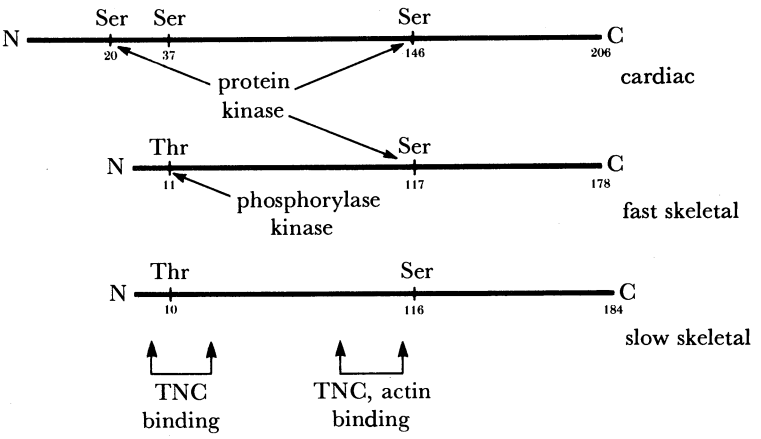
Tropomyosin

The α subunit of tropomyosin has been shown to be phosphorylated in the penultimate residue, serine 283 of rabbit and frog skeletal muscles (Mak *et al.* 1978). Phosphorylation is not confined to the α subunit, because phorphorylated forms of the β and other isotypes of tropomyosin that have been detected, particularly in slow skeletal muscle (see Heeley *et al.* 1983), have also been reported (O'Connor *et al.* 1979; Heeley *et al.* 1982, 1983). In adult skeletal muscle the extent of phosphorylation is relatively low, about 10–20 % in the case of the α and β subunits (Heeley *et al.* 1982). Levels are much higher in foetal and early post-natal striated and cardiac muscles. In the rat heart, for example, 7 days before birth 70 % of the α subunit, the only isotype detectable in this tissue, is phosphorylated (Heeley *et al.* 1982). These results suggest that the increased phosphorylation is associated with the active myofibrillogenesis that will be occurring in the rapidly developing muscle of the foetus and immediately after birth. Such an association would be expected if phosphorylation of tropomyosin subunits plays a part in the regulation of filament formation. As there is evidence that the linear aggregation of tropomyosin involves an overlap of 8 or 9 residues between the N and C terminal ends of the molecule, phosphorylation of the serine 283 would produce a marked change in charge in this region. This could alter the stability of the polymer, as has been suggested by Mak *et al.* (1978). In adult frog striated muscle at least there is no evidence that the level of tropomyosin phosphorylation changes on stimulation (Barany & Barany 1977). Indeed, the evidence available suggests that the turnover of the tropomyosin-bound phosphate is less rapid than that of the P light chain of myosin or cardiac troponin I, implying that it may have a more passive or structural role.

Troponin I

The role of phosphorylation in the regulation of contractile activity is most clearly understood in relation to the function of the troponin I in cardiac muscle. This protein, the component of the troponin complex that inhibits the Mg^{2+}-stimulated ATPase of actomyosin has sites that can be phosphorylated by cyclic-AMP-dependent kinase and phosphorylase kinase. These sites

have been identified in troponin I from fast skeletal muscle (Moir *et al.* 1974; Huang *et al.* 1974) and from heart (Moir & Perry 1977, 1980; Moir *et al.* 1980). Although troponin I from fast and slow skeletal and cardiac muscles are all different gene products, these proteins exhibit considerable homology, particularly in the troponin C-binding region close to the N-terminus, residues 1–21, and in the troponin C and actin-binding region, residues 96–116, in the fast skeletal form from rabbit muscle (Wilkinson & Grand 1978). Threonine 11 is the preferred site of phosphorylation catalysed by phosphorylase kinase and serine 117 by cyclic-AMP-dependent kinase (Moir *et al.* 1974; Huang *et al.* 1974). The former lies within the N-terminal troponin C-binding site whereas the latter phosphorylation site is very close to the other troponin C-binding site (figure 1). The proximity of the phosphorylation sites to the regions of interaction



representation of the primary sequences of fast and slow skeletal and cardiac mus
the rabbit. N, N terminus; C, C terminus. The serine and threonine residues identified are
ve phosphorylation sites. TNC, troponin C.

with troponin C is probably the reason why in the presence of this protein phosphorylation of both sites is almost completely inhibited. As the sites on troponin I involved in interaction with troponin C are positively charged, phosphorylation at threonine 11 and serine 117 would markedly effect the net charge in these regions and presumably in some way change the nature of the interaction with troponin C, an interaction that at least in part would be expected to be electrostatic in nature. The corresponding regions for interaction with troponin C of troponin I from slow skeletal and cardiac muscles are virtually identical in amino acid sequence with serine residues in homologous positions to threonine 11 and serine 117 of fast skeletal troponin I. This implies that these regions are of particular significance for the functioning of the troponin complex. Nevertheless the role of phosphorylation at the two major sites on fast skeletal troponin I is far from clear. In the frog injected with ^{32}P-labelled inorganic phosphate little incorporation of radioactivity was detected in troponin I isolated from skeletal muscle (Ribolow *et al.* 1977). On the other hand, troponin I isolated from fast and slow skeletal muscles of the rabbit contains significant amounts of covalently bound phosphate when isolated by affinity chromatographic methods from fresh muscle under conditions in which the endogenous phosphatase activity is inhibited (Cole & Perry 1975). This endogenous phosphate is presumably located at the sites on troponin I that can be phosphorylated by cyclic-AMP-dependent protein kinase or phosphorylase kinase *in vivo* but this aspect has not been investigated.

Although phosphorylation of skeletal troponin I has yet to be shown to be of physiological significance, a well defined role has been identified for the process in cardiac muscle. In addition to containing sites homologous to those found in the fast skeletal form of troponin I, the cardiac protein contains an additional cyclic-AMP protein kinase-specific site at serine 20. This residue is located on the 26 residue N-terminal peptide that is unique to cardiac troponin I (figure 1). Owing to the presence of this site, cardiac troponin I is a much better substrate for cyclic-AMP protein kinase *in vitro* than the skeletal form. Troponin I from the rabbit heart also contains more covalently bound phosphate than the skeletal form when isolated directly from the tissue by affinity chromatography. When the perfused heart is treated with adrenalin the level of phosphorylation of the troponin I increases (England 1975; Solaro *et al.* 1976). In the rabbit the extent of phosphorylation of the serine 20 of troponin I changes from 20–30 % in the normal perfused heart to 90–100 % during the inotropic response to adrenalin (Moir *et al.* 1980). Although it has been reported that in the rat heart the increase in phosphorylation of troponin I correlates with the rise in force (England 1975), this does not appear to be so in the rabbit heart, where phosphorylation reaches a maximum some time after the developed force has reached its peak (Westwood & Perry 1981). It was originally considered that the role of phosphorylation of troponin I was directly related to the development of increased force by increasing the sensitivity of actomyosin ATPase to Ca^{2+} (Rubio *et al.* 1975) but it was later reported that phosphorylation made the system less sensitive to Ca^{2+}, i.e. the pCa^{2+} required for half-maximal ATPase activity fell with increasing phosphorylation of serine 20 of troponin I (Solaro *et al.* 1976; Ray & England 1976; Reddy & Wyborny 1976). Thus phosphorylation of serine 20 of cardiac troponin I is a special adaptation to modulate excitation–contraction coupling in the heart. Unlike skeletal muscle, in which development of increased force occurs by recruitment of fibres with the speed of contractile response of each cell type remaining constant, cardiac muscle can simultaneously increase the force developed by each cell, and change the time course of the response. Stimulation of the adrenergic receptors results in an increase both in Ca^{2+} flux and the extent of phosphorylation of troponin I. The latter covalent modification, by decreasing the sensitivity of the actomyosin ATPase to Ca^{2+}, constitutes a negative feedback system that helps to stabilize the response and results in a more rapid shut-off of the ATPase when the Ca^{2+} concentration falls during relaxation. This explains the speeding up of relaxation that is associated with the positive inotropic effect of adrenalin on cardiac muscle.

It is of interest to speculate how phosphorylation of cardiac troponin I at serine 20 can change the metal-binding properties of troponin C that is reflected in the change in sensitivity of the actomyosin ATPase to Ca^{2+}. Some hints about the mechanism can be obtained by extrapolation from the results of investigations on the interaction between skeletal forms of troponin C and troponin I. In view of the strongly conserved nature of the troponin C-binding regions of skeletal and cardiac troponin I and the very marked homology between the different forms of troponin C (slow skeletal and cardiac troponin C are identical in amino acid sequence), there is considerable justification for this approach. A number of investigations, particularly those employing proton magnetic resonance, have identified the amino acid residues in both proteins that are involved in the interactions (Weeks & Perry 1977, 1978; Leavis *et al.* 1978; Evans & Levine 1980; Grabarek *et al.* 1981; Grand *et al.* 1982*a*; Dalgarno *et al.* 1983). There are also suggestions from several lines of evidence that the binding of Ca^{2+} to the two N-terminal binding sites of troponin C, the so-called sites I and II, is the event that results in switching on the ATPase of the actomyosin. In cardiac muscle troponin C site I is imperfect in that the amino

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acid sequence does not conform to the requirements of a Ca^{2+} binding site of high specific affinity (Kretzinger 1980). Thus the Ca^{2+} binding characteristics of site II occupying the region encompassed by residues 63 to 74 in rabbit cardiac muscle troponin I determine the response of cardiac actomyosin ATPase. The phosphorylation of serine 20 of troponin I is not blocked

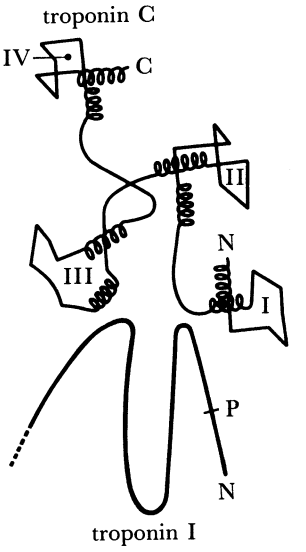

FIGURE 2. Schematic representation of the interaction between troponin I and troponin C of cardiac muscle. Data obtained from n.m.r. studies on the interaction between fast skeletal forms of the troponin components (Dalgarno *et al.* 1982). I, II, III and IV are sites of Ca^{2+} binding. Site I is not an effective binding site in cardiac muscle and site II is probably the regulatory site. N, N terminus; C, C terminus. P is the phosphorylation site at serine 20 of cardiac troponin I.

in the presence of troponin C and presumably is not directly involved in the interaction between the two proteins. Nevertheless, the phosphorylation of this site must effect the interaction in order to change the Ca^{2+}-binding properties of site II on the troponin C. Preliminary evidence of a change of affinity for troponin C as a consequence of phosphorylation of troponin I from cardiac muscle has been obtained from electrophoretic and enzymic studies (M. Ohnishi & S. V. Perry, unpublished results). The precise mechanism causing this effect can only be a matter of speculation at the moment, but presumably it involves changes in the troponin-C–tropinin-I interaction (figure 2). On general theoretical grounds it is likely that the decrease in affinity for Ca^{2+} suggested by the enzymic studies is the result of an increase in the off rate for binding by this cation at site II. Recent studies by Robertson *et al.* (1982) suggest that this is indeed so.

REGULATION OF A FILAMENT

Myosin phosphorylation is the most extensively studied of the myofibrillar systems and probably the best defined in terms of the enzymes involved. Attention here will be confined to the enzyme myosin light-chain kinase, an extremely specific enzyme that catalyses the phosphorylation of the light chain of myosin of molecular mass 18–20 kDa known as the dithiobisnitrobenzoic acid (DTNB), the regulatory or P light chain. The site of phosphorylation in rabbit fast skeletal muscle is serine 14 or 15, probably the latter because serine 14 is absent in the homologous light chain of myosin from molluscan adductor muscle. This muscle is unusual

in that the light-chain kinase is apparently absent, and the regulatory light chain is not phosphorylated by added kinase (Frearson *et al.* 1976; Kendrick-Jones & Jakes 1977). Although in the higher animals phosphorylation is restricted to the P light chain, evidence has been obtained for phosphorylation of the myosin heavy chain, which can effect the ATPase activity in *Acanthamoeba* (Maruta & Korn 1977; Korn 1978).

In addition to their high specificity for the P light chain, different forms of the myosin light-chain kinase exist in different muscle types. A highly specific myosin light-chain phosphatase has also been isolated from rabbit skeletal muscle (Morgan *et al.* 1976), but although a number of protein phosphatases that can dephosphorylate the P light chain have been identified in vertebrate smooth muscle, no enzyme comparable in molecular mass and specificity with that isolated from rabbit fast skeletal muscle has yet been reported (Pato & Adelstein 1980).

The wide distribution of myosin light-chain kinase, both in muscle and non-muscle systems and the specificity of the enzyme, implies that it has a special role in myosin function. It is a striking fact that although phosphorylation of myosin can be demonstrated with all myosins apart from that of molluscan adductor muscle, the reported effects *in vitro* associated with P light-chain phosphorylation are by no means the same with all types of myosin. Whereas phosphorylation of the P light chain appears to be essential for actin activation of the ATPase of smooth muscle myosin, this is not true of myosins from striated muscle (Morgan *et al.* 1976). Phosphorylation also appears to play a role in the thick filament formation by smooth muscle myosin (Suzuki *et al.* 1978, 1982; Kendrick-Jones *et al.* 1982; Trybus *et al.* 1982; Onishi 1982; Onishi & Wakabayashi 1982), but as yet there are no reports of a role for the process in the formation of A filament in striated muscle.

Striated muscle

Neither phosphorylation nor dephosphorylation of the P light chain of myosins from skeletal and cardiac muscle is essential for ATPase activity. Clearly the role of phosphorylation in regulating the ATPase activity of myosin or actomyosin from striated muscle is not similar to that involved in enzymes such as phosphorylase or glycogen synthase. Although not essential for ATPase activity, phosphorylation of the P light chain of striated muscle myosin has been reported to produce a slight, barely significant increase (Morgan *et al.* 1976; Perry *et al.* 1978). Under certain conditions the effect is reported to be more pronounced (Pemrick 1980).

In view of the role of phosphorylation in filament formation in smooth myosin in particular, it is possible, however, that the state of aggregation of the myosin is an important factor in determining the effect of light-chain phosphorylation on actomyosin ATPase. In this respect the recent report (Cooke *et al.* 1982), that when myosin is fixed in the myofibrils by previous mild treatment with glutaraldehyde, phosphorylation of the P light chain produces a decrease in actomyosin ATPase, is of interest. With the uncertainties that exist about the role of phosphorylation on the *in vitro* systems, it is obviously difficult to speculate on a role for the process *in vivo*.

The myosin light-chain kinase system consists of a catalytic subunit of molecular mass about 80 kDa (Pires & Perry 1977) and calmodulin (Yagi *et al.* 1978; Nairn & Perry 1979), which is activated by Ca^{2+} concentrations similar to those that activate the myofibrillar ATPase. Thus on the stimulation of muscle the kinase will be activated, leading to phosphorylation of the P light chain. From studies of the yields and specific activities of the isolated enzyme it is clear even in fast skeletal muscle, in which tissue the kinase is most active, that the phosphorylation

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and dephosphorylation of P light chain cannot occur synchronously with the cross-bridge cycle. In fast skeletal muscle of the rabbit the maximum myosin ATPase activity per unit mass of muscle is estimated to be about 50–100 times greater than the kinase. The discrepancy is even greater in cardiac muscle, which has a much lower kinase activity. Further, in skeletal muscle the light-chain phosphatase activity is lower than that of the kinase, thus increasing the discrepancy between the rate of ATP hydrolysis by the ATPase and the phosphorylation–dephosphorylation cycle.

To assess the response of the phosphorylation system to contractile activity it is necessary to determine the levels of phosphorylation in resting muscle. In most resting striated muscles the P light chain is partly phosphorylated. There is some variation in the reports in the literature as to the resting level, which may depend upon species and types of muscle used and whether it is determined on the isolated muscle or in the intact animal (Stull & High 1977; Barany *et al.* 1979, 1982; Manning & Stull 1977; Kushmerick & Crow 1982). The results obtained in our laboratory with rabbit muscles frozen *in situ* by Wollenberg clamps are listed in table 2.

In the rabbit heart, the level of P light-chain phosphorylation is relatively low, about 23 %, whether in the whole animal or the isolated perfused heart. Similar values have been obtained from other species. Most reports (Perry *et al.* 1978; Westwood & Perry 1981; Holroyde *et al.* 1979; Jeacock & England 1980; Kranias & Solaro 1982; cf. Kopp & Barany 1979) indicate little, if any, significant change in phosphorylation when the contractile activity of the heart is increased by intervention with adrenalin. On the other hand, when determined simultaneously in the same heart the phosphorylation of troponin I increases (Westwood & Perry 1982), indicating that in cardiac muscle phosphorylation of the P light chain, unlike troponin I, is not under β-adrenergic control.

On stimulation of fast muscles of the rabbit the extent of phosphorylation of the P light chain increases, rising to virtually 100 % after 5 s isometric tetanus in the rabbit (Westwood *et al.* 1982). Similar values have been obtained with isolated rat fast muscles after periods of isometric tetanus (Manning & Stull 1979). On the other hand, with isolated chicken muscles even after prolonged tetanus, phosphorylation of the P light chain did not exceed 50 % (Barany *et al.* 1982). In general, the frequency of stimulation is more effective in promoting phosphorylation than the number of impulses, suggesting that the effective average calcium concentration of the sarcoplasm is more important, rather than the maximum transient levels for activation of the kinase (Westwood *et al.* 1982, 1983). After phosphorylation the rate of dephosphorylation is relatively slow in the intact rabbit; as has also been reported for isolated rat skeletal muscle (Manning & Stull 1979) and muscle extracts (Fearson *et al.* 1976). Tetanic stimulation of rabbit soleus muscle increased the phosphorylation up to more than 80 %, but longer periods of repeated stimulation were required to obtain similar levels of phosphorylation to those in fast skeletal muscle (Westwood *et al.* 1983).

The studies on whole muscle in this laboratory suggest that increased phosphorylation of the P light chain is a feature of continued contractile activity in both fast and slow skeletal muscle. The inability by some authors (e.g. Kushmerick & Crow 1982) to show P light-chain phosphorylation in mammalian slow skeletal muscle is difficult to explain, but may reflect species differences or technical difficulties in the measurements. The observations suggest that phosphorylation is a mechanism for modulating the contractile response and one that is most strongly expressed in fast skeletal muscle, which contains the most active kinase. It has been proposed by Manning & Stull (1979) that P light-chain phosphorylation may play a role in post-tetanic

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potentiation, based on the correlation between these two phenomena observed in rat fast skeletal muscle. In the combined rabbit extensor digitorum longus and tetanus anterior muscles, however, there is not a good correlation between the decay of P light-chain phosphorylation and P light-chain phosphorylation after tetanus (Westwood *et al.* 1982, 1983). The fact also that significant P light-chain phosphorylation occurs in rabbit soleus muscle, which does not exhibit post-tetanic potentiation (Close & Hoh 1969), suggests that these two properties are not very closely related. On the other hand, Kushmerick & Crow (1982) consider that P light-chain phosphorylation is responsible for the fall in maximum velocity of shortening that occurs after tetanus. This role would require that phosphorylation of the P light chain produces a fall in the V_{max} of the actin-activated ATPase. Although studies *in vitro* with isolated myosin are not compatible with this hypothesis, it is supported by the recent reports of the inhibition of the ATPase by phosphorylation of cross-linked myofibrils (Cooke *et al.* 1982).

TABLE 2. LEVELS OF PHOSPHORYLATION OF THE P LIGHT CHAINS OF MYOSIN IN RESTING RABBIT STRIATED MUSCLES *IN VIVO*

muscle	type	percentage phosphorylation	kinase activity
skeletal†			
combined extensor digitorum longus/ tibialis	fast	50.4 ± 3.4 ($n = 11$)	high
rectus abdominis	fast	54.8 ± 2.6 ($n = 4$)	high
soleus	slow	17.2 ± 1.0 ($n = 6$)	medium
cardiac‡		23.0 ± 3.0 ($n = 4$)	low

† From Westwood *et al.* (1982, 1983).
‡ From Westwood & Perry (1981), normal beating heart in the anaesthetized animal.

Smooth muscle

Myosin light-chain phosphorylation appears to have a role in vertebrate smooth muscle that is different from that in striated muscle. Phosphorylation of smooth myosin affects the conformation adapted by the tail of the molecule with respect to the head and thus changing its sedimentation characteristics and its ability to form filaments (Suzuki *et al.* 1978, 1982; Onishi & Wakabashi 1982; Kendrick-Jones *et al.* 1982; Trybus *et al.* 1982). Apart from consistent reports from Ebashi's laboratory, most investigators report that the actin activation of the Mg^{2+} ATPase of vertebrate smooth muscle myosin is very low if it is unphosphorylated. Treatment with kinase and tissue extracts containing the enzyme increases the Mg^{2+}-stimulated actomyosin ATPase. Usually the most active preparations are the most highly phosphorylated. It is difficult, however, in some cases to assess the significance of the activation reported by different workers using what appear to be roughly comparable assay conditions. Fully activated vertebrate smooth muscle actomyosin has been reported by some workers with specific activities ranging from 30–50 nmol min^{-1} mg^{-1} (Persechini & Hartshorne 1981) to 200–300 nmol min^{-1} mg^{-1} (Sobieszek 1977) and in some cases exceptionally as high as 600–800 nmol min^{-1} mg^{-1} (H. A. Cole & S. V. Perry, unpublished observations). This extremely wide range, which appears to be a feature of smooth muscle myosin, is not found with the skeletal muscle enzyme and may reflect the complexity of the activation process of smooth muscle. It certainly presents difficulties in deciding upon the physiological significance of the effects described at the lower end of the range of activities reported.

Smooth muscle contains no troponin of the type found in skeletal muscle but does contain

relatively large amounts of calmodulin (Grand & Perry 1979), and despite efforts from a number of laboratories troponin C has not been isolated. Ebashi and collaborators, however, consider that phosphorylation is not essential for activation and that additional factors, leiotonin A and C, are required (Mikawa *et al.* 1977). Leiotonin C, although not well characterized, appears to be similar to, but not identical with, troponin C or calmodulin.

In view of the evidence, the simplest view would be that actin activation of the smooth muscle myosin ATPase requires phosphorylation of the P light chain. According to this scheme, contraction is triggered by activation of the light-chain kinase by the binding of Ca^{2+} by calmodulin, and relaxation would follow as a consequence of dephosphorylation by the light-chain phosphatase (figure 3).

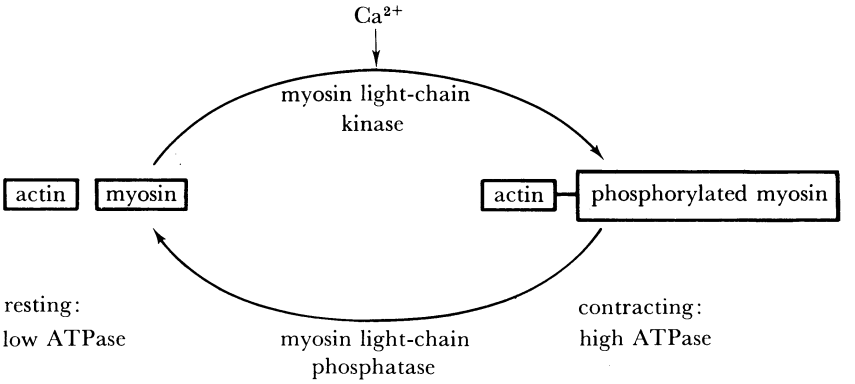

FIGURE 3. Scheme for the regulation of the Mg^{2+} ATPase of smooth muscle actomyosin by phosphorylation of the P light chain of myosin.

Such a mechanism requires that in resting smooth muscle the P light chain is completely dephosphorylated but is rapidly phosphorylated on stimulation. Recent studies by Somlyo *et al.* (1981) indicate that this is so, although other studies have indicated that in some resting smooth muscles, as with skeletal muscle, there may be a significant degree of phosphorylation (Barron *et al.* 1979). Some phosphorylation could occur, however, without activation of the ATPase if both heads must be phosphorylated for activation (Persechini & Hartshorne 1981; Ikebe *et al.* 1982). If one of the heads were preferentially phosphorylated by the enzyme owing to co-operative effects (Persechini & Hartshorne 1981), significant levels of phosphorylation would be detected without activation of the actomyosin ATPase.

Although such a scheme has a number of attractive features there are suggestions that activation is more complex than a simple phosphorylation–dephosphorylation mechanism. In the first place, although phosphorylation occurs on stimulation of smooth muscle, the level is not maintained even though tension is (Dillon *et al.* 1981; Butler & Siegman 1981; Butler *et al.* 1982). This indicates that the cross-bridge cycling required to maintain tension may not be directly related to the level of phosphorylation.

Studies of the actomyosin ATPase *in vitro* suggest that a factor in addition to phosphorylation of the light chain, which is also calmodulin-dependent, can increase activity (Cole *et al.* 1982). This effect can be obtained with fully phosphorylated myosin, and variable contamination with it may account, at least in part, for the wide range of actomyosin ATPase activities reported in the literature. It is widely agreed that the increase in ATPase activity is not linearly related to the extent of phosphorylation. This property of the system is explained by some workers on

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the basis of the cooperative effect referred to above and the requirement for both heads of the molecule to be phosphorylated for activation of the myosin ATPase. Nevertheless, reports still appear claiming that phosphorylation alone is not sufficient to activate some preparations fully (Cole *et al.* 1982; Merkel *et al.* 1982; Persechini *et al.* 1981).

In studies in which the enzymic activity was carefully monitored during the course of the light-chain phosphorylation in the presence of added endogenous or kinase, the rate of ATPase activity remained steady even though the extent of phosporylation changed from 30 to 80% (Cole *et al.* 1980, 1982). Similar unchanged rates of ATPase activity were observed during dephosphorylation in the presence of an inhibitor that possessed phosphatase activity (Grand *et al.* 1982 *b*). It is very difficult to reconcile these results with the observation that the most active preparation of smooth muscle actomyosin are the most highly phosphorylated. Correlation between enzymic activity and level of phosphorylation has consistently been reported in other systems such as pyruvate dehydrogenase, a classical example of an enzyme regulated by phosphorylation. The reasons for the inconsistencies in the results reported by workers on the smooth myosin system is not clear. A number of possibilities exist. It is conceivable that the state of aggregation of the actomyosin system is important in determining the ATPase activity, an aspect that possibly has not been rigorously enough controlled in all the enzymic assays reported. Another explanation is that control of ATPase activity by phosphorylation is a very sensitive property of the myosin (or actin) and that this property is modified as a consequence of subtle changes that can occur in handling the preparation.

TABLE 3. EFFECT OF PHOSPHORYLATION ON THE Ca^{2+} SENSITIVITY OF THE SYSTEMS THAT MODULATE THE Mg^{2+} ATPASE OF MUSCLE ACTOMYOSIN

protein system			Ca^{2+} sensitivity†
calcium-binding protein	phosphorylated protein	muscle type	after phosphorylation
troponin C	troponin I	cardiac	rises
calmodulin	myosin light-chain kinase catalytic unit	smooth	rises
myosin heavy chain (?)	P light chain	smooth	falls

† $[Ca^{2+}]$ required for 50% activation of the ATPase.

Clearly it appears that myosin light-chain kinase has a function in smooth muscle that is different from its role in striated muscle. Quite apart from the activating effect on the actomyosin ATPase, regulation of the light-chain kinase itself in smooth muscle has features unique to this tissue. The smooth muscle light-chain kinase catalytic unit has a higher molecular mass than the striated muscle enzyme (Adelstein & Klee 1981), and its activity can be changed by phosphorylation, an effect for which there is as yet no evidence in striated muscle kinase. Phosphorylation by cyclic-AMP-dependent protein kinase of sites on the catalytic unit in smooth muscle kinase results in a lowered affinity for calmodulin. Thus the action of adrenalin would be expected to lead to a lowered kinase and presumably ATPase activities for a given intracellular Ca^{2+} concentration (Adelstein *et al.* 1980).

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Conclusions

From studies of the well characterized phosphorylation systems of the myofibril that modulate the ATPase, and hence the contractile process, certain generalizations can be made. All three systems to which reference has been made possess certain common features (table 3). One component of the system is a specific calcium-binding protein that forms a complex with the other protein, which may or may not be an enzyme. Binding between the proteins in two cases, and probably in all three, is increased in the presence of Ca^{2+} and the interaction affects the binding constants of the sites on the calcium binding protein. Phosphorylation of the protein that does not bind calcium changes the characteristics of the binding sites on the other protein to modify the response of the intact system to a given Ca^{2+} concentration. Thus phosphorylation followed by dephosphorylation enables the rate of hydrolysis of ATP by the actomyosin at a given Ca^{2+} concentration to change in a reversible manner and thus modulates the contractile response. The systems described are strongly expressed in muscle with its rapid, highly specific response to changes in Ca^{2+} flux. It is likely that a similar role of phosphorylation will be found to be associated with other processes in the cell that are regulated by calcium.

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