CALCIUM REGULATION OF MUSCLE CONTRACTION

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ABSTRACT Calcium triggers contraction by reacting with regulatory proteins that in the absence of calcium prevent interaction of actin and myosin. Two different regulatory systems are found in different muscles. In actin-linked regulation troponin and tropomyosin regulate actin by blocking sites on actin required for complex formation with myosin; in myosin-linked regulation sites on myosin are blocked in the absence of calcium. The major features of actin control are as follows: there is a requirement for tropomyosin and for a troponin complex having three different subunits with different functions; the actin displays a cooperative behavior; and a movement of tropomyosin occurs controlled by the calcium binding on troponin. Myosin regulation is controlled by a regulatory subunit that can be dissociated in scallop myosin reversibly by removing divalent cations with EDTA. Myosin control can function with pure actin in the absence of tropomyosin. Calcium binding and regulation of molluscan myosins depend on the presence of regulatory light chains. It is proposed that the light chains function by sterically blocking myosin sites in the absence of calcium, and that the "off" state of myosin requires cooperation between the two myosin heads. Both myosin control and actin control are widely distributed in different organisms. Many invertebrates have muscles with both types of regulation. Actin control is absent in the muscles of molluscs and in several minor phyla that lack troponin. Myosin control is not found in striated vertebrate muscles and in the fast muscles of crustacean decapods, although regulatory light chains are present. While in vivo myosin control may not be excluded from vertebrate striated muscles, myosin control may be absent as a result of mutations of the myosin heavy chain.

GENERAL FEATURES

Contraction of muscles is triggered by calcium. At rest, the concentration of calcium ions in the millieu surrounding the contractile proteins is less than about 10^{-8} M. The low concentration of intracellular calcium ions is maintained by the action of the "calcium pump" that sequesters calcium ions within the membraneous compartments of the sarcoplasmic reticulum in striated muscles, within vesicles in smooth muscles and perhaps across the plasma membranes in some instances (Hasselbach and Makinose, 1961; Ebashi and Lipman, 1962). Stimulation initiates contraction by releasing calcium from the sarcoplasmic reticulum and raising its concentration in the sarcoplasm to about 10^{-6} M. Once stimulation is over, calcium ions are sequestered again within the sarcoplasmic reticulum and the resting state is reestablished (Hasselbach,

1964; Ebashi and Endo, 1968). Although calcium ions are the only known in vivo triggers of contraction, the way calcium participates in regulation may vary in different muscles. In general, there are two kinds of regulation: one type associated with actin, the other with myosin.

The evidence is overwhelming that movement and tension generation are the result of a direct interaction between myosin and actin (Huxley, 1969). At rest, i.e. in the presence of ATP, cross-links are not made between these two proteins, the muscle stretches readily, and the actin filaments may be pulled out with ease from the array of the myosin filaments. The resting state is maintained by preventing interaction between myosin and actin, and the role of calcium is to remove this inhibition.

Pure rabbit actin and myosin are not regulated by calcium ions; these proteins combine readily even in the absence of calcium ions. Calcium controls function only in the presence of additional components. These components are the regulatory proteins; they convert actomyosin into a calcium sensitive system. Their effect is to prevent actomyosin formation in the absence but not in the presence of calcium. Regulatory proteins were discovered by Ebashi (1963) in rabbit muscle and shown to consist of troponin and tropomyosin (Ebashi and Kodama, 1965). Troponin and tropomyosin regulate by blocking sites on actin in the absence of calcium, thereby preventing its interaction with myosin in the presence of Mg-ATP (Weber and Murray, 1973). A different regulatory system operates in molluscan muscles (Kendrick-Jones et al., 1970). In these muscles the regulatory component is a subunit of myosin. Here the regulatory light chain blocks sites on myosin, thereby preventing its interaction with actin in the absence of calcium (Szent-Györgyi et al., 1973). The control elements are different in the two types of regulation. They act on different proteins and the detailed mechanism of their action is different. Nevertheless, the overall effect of both regulatory systems is the same; both interfere with actomyosin formation in the presence of Mg-ATP, and the inhibition of complex formation in both regulations is reversed by small amounts of calcium.

The major features of actin control will be discussed first. Then I will review the main features of myosin control that my colleagues, John Kendrick-Jones, William Lehman, and Eva M. Szentkiralyi, and I have described. I will summarize the results of our comparative studies, and show how the two regulatory systems are distributed in the animal kingdom, and will speculate on possible mechanisms of how myosin control may operate.

In this discussion contractile activity will be deduced from ATPase activity. The magnesium activated ATPase activity of myosin is stimulated by actin. This stimulation is the result of actomyosin formation, is related to the cross-bridge cycle, and requires calcium ions provided regulatory proteins are present. The measure of the calcium dependence of the Mg-ATPase of an actomyosin preparation is obtained from ATPase activities in the absence and in the presence of calcium. Calcium sensitivity equals $(1 - [\text{ATPase EGTA}/\text{ATPase Ca}]) \times 100$. Calcium binding and calcium sensitivity are always measured in the presence of millimolar concentrations of magnesium.

The great excess of magnesium swamps non-specific effects of divalent cations and insures that the control functions attributed to calcium are specific ones.

ACTIN CONTROL

Four different proteins are involved in regulating actin sites: tropomyosin and the three subunits of troponin (Greaser and Gergely, 1971). These regulatory components interact with actin and with each other in a very specific manner. Tropomyosin is a rodlike molecule of about 65,000 daltons. It consists of two α-helices wound into a coiled-coil having a length of about 400 Å; the molecule bonds head to tail to form polar filaments (Caspar et al., 1969). The molecular weight of the troponin complex in rabbit is about 80,000 daltons; its three different subunits have different functions. There is an 18,000 dalton calcium binding component, troponin-C, that binds calcium with high affinity and undergoes calcium dependent changes in conformation. A 23,000 dalton component, troponin-I, has an inhibitory function, while a 37,000 dalton component TN-T binds to actin (Hartshorne and Mueller, 1968; Schaub and Perry, 1969; Greaser and Gergely, 1971).

Troponin and tropomyosin together with actin are the major components of the thin filaments (Ebashi et al., 1969; Lehman et al., 1972) (Fig. 1). Troponin is distributed periodically with one troponin complex every 385 Å on each of the actin strands (Ohtsuki et al., 1967). Troponin is globular and each molecule is separated by a distance of several hundred angstroms on the thin filaments. This means that most of the actin monomers that form the thin filaments are not in direct contact with troponin. Since troponin is the only component of the thin filament that can interact with calcium at the physiological calcium and magnesium concentrations, the problem arises as to how troponin can influence the reactivity of all the actin monomers. Moreover, studies indicate that all the actin monomers are able to react with myosin (Moore et al., 1970). The binding of calcium on one troponin-C results in switching about 7 actin monomers from the "off" state to the "on" state, so that the actin monomers function in a cooperative manner (Bremel and Weber, 1972).

The changes in the state of troponin are communicated to distant actin monomers via tropomyosin (Ebashi et al., 1969). Tropomyosin lies in the grooves formed by the two strands of actin (Hanson and Lowy, 1963; O'Brien et al., 1971; Spudich et al.,

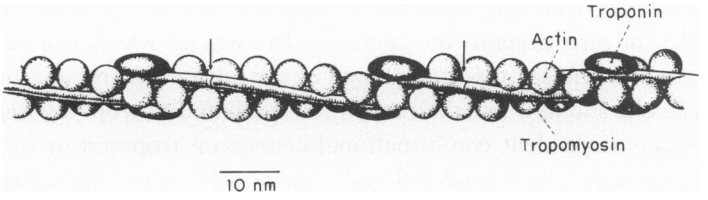

FIGURE 1 A model for the fine structure of the thin filament. (From Ebashi et al., 1969, modified by Ohtsuki, 1974.)

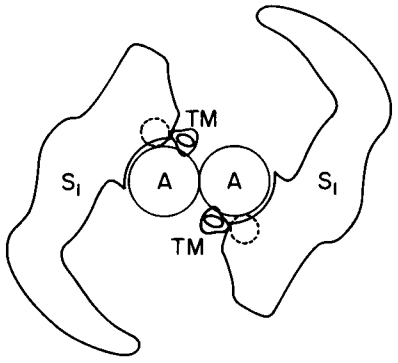

FIGURE 2 Composite end-on view of actin-tropomyosin-S_1 structure. The shape of the S_1 subunits and the position at which they attach to the actin is copied directly from plate IX of Moore et al. (1970). The two tropomyosin (TM) positions correspond to: dotted contours, activated state; solid contours, relaxed state, based on radial position of reflection on third layer line of relaxed muscle. The possible way in which tropomyosin could block the attachment of a cross bridge is very evident. (From Huxley, 1972.)

1972). Troponin is positioned on the thin filaments by tropomyosin and is located about 130 Å from the end of each tropomyosin molecule (Cohen et al., 1972; Ohtsuki, 1974). X-ray diagram of muscle shows that the exact position of tropomyosin within the grooves of the thin filaments depends on the state of troponin; intensity changes during contraction and rigor indicate that tropomyosin moves towards the center of the groove (O'Brien et al., 1971; Haselgrove, 1972; Huxley, 1972; Lowy and Vibert, 1972; Vibert et al., 1972; Parry and Squire, 1973) (Fig. 2). Three-dimensional reconstruction of electron microscopic images of thin filaments suggests that at rest tropomyosin is situated somewhat away from the center of the actin groove and positioned in a way that it is likely to interfere with the binding of the S-1 portion of myosin (Moore et al., 1970; Spudich et al., 1972; Huxley, 1972).

The simplest picture at present of regulation by actin is the assumption that the actin sites are blocked sterically by tropomyosin, and that tropomyosin has at least two states (Huxley, 1972; Parry and Squire, 1973). The "off" state is characteristic of resting muscles, and is the one that depends on troponin being free of calcium. The "on" state is the position of tropomyosin in the absence of troponin, or its position when troponin is saturated with calcium. Contraction or rigor is made possible by the movement of tropomyosin, a movement that may be governed by the conformation of troponin which in turn depends on calcium. The way in which troponin regulates tropomyosin movement is not clear and some of the salient features are just emerging. Interactions among subunits and their relation to actin and to tropomyosin depend on calcium. Calcium-dependent conformational change on troponin or on troponin-C has been demonstrated by several different optical techniques, including changes in circular dichroism (Murray and Kay, 1972); in absorbency (Head and Perry, 1974); in fluorescence (van Eerd and Kawasaki, 1973); and in electron spin resonance (Potter et al., 1974; Ebashi et al., 1974). The subunits of troponin appear to be linked in a

serial manner, so that troponin-I interacts with actin and troponin-C; troponin-C combines with troponin-I and troponin-T; troponin-T combines with troponin-C and tropomyosin (Margossian and Cohen, 1973; Potter and Gergely, 1974; Hitchcock, 1975). The interactions between the troponin subunits and their affinity to actin and tropomyosin depends on changes in calcium concentration in the physiological range. The complex troponin-I and troponin-C binds to actin-tropomyosin only in the absence of calcium (Hitchcock and Szent-Györgyi, 1973; Hitchcock et al., 1973; Potter and Gergely, 1974). The association of troponin-C with troponin-T is strengthened by calcium (Ebashi et al., 1972; Margossian and Cohen, 1973). The interpretation of the changes await further structural studies. Determination of the amino acid sequence of troponin-C (Collins et al., 1973) and its recent crystallization (Mercola et al., 1975) open the way towards elucidating its three-dimensional structure which will represent a major step in our understanding of troponin function.

One notes that the main features of actin-control, including the requirement for tropomyosin and the multiple subunit nature of troponin, with clearly identifiable functions, are exhibited also in invertebrate species, such as insects (Bullard et al., 1973) and lobster (Regenstein and Szent-Györgyi, 1975), although various troponins may differ in the size of the subunits and in calcium binding. The state requiring troponin is the relaxed state of muscle, where tropomyosin is found in an inhibitory position; contraction cannot take place since actin and myosin cannot form a complex. The reduced affinity of regulated actin to myosin in the absence of calcium is borne out by the kinetic studies of the ATPase activity (Eisenberg and Kielley, 1970; Hartshorne and Pyun, 1971; Koretz et al., 1972). The components involved in actin regulation are confined to the thin filaments. Troponin or tropomyosin do not combine with myosin and their effect is restricted to actin and to tropomyosin.

MYOSIN CONTROL

Evidence for Myosin Control

The discovery of myosin control was a by-product of efforts to prepare thin filaments in their native state without exposing the preparations to organic solvents or to concentrated salt solutions. The first successful thin filament preparations were obtained from the adductor muscle of the clam, *Mercenaria mercenaria* (Szent-Györgyi et al., 1971). The thin filaments of the clam complexed with rabbit myosin; however, the ATPase activity of the hybrid actomyosin was not calcium sensitive (Fig. 3). This was a surprising finding which seemed to contradict the notion based on studies of vertebrate muscles, that the regulatory components of muscle are located on the thin filaments. One had to assume either that regulatory proteins were lost from the thin filament preparation, or that regulation in molluscs differed from regulation in vertebrate muscles (Szent-Györgyi et al., 1971). The puzzle was solved by demonstrating that in molluscan muscles regulation is associated with myosin and not with the thin filaments (Kendrick-Jones et al., 1970).

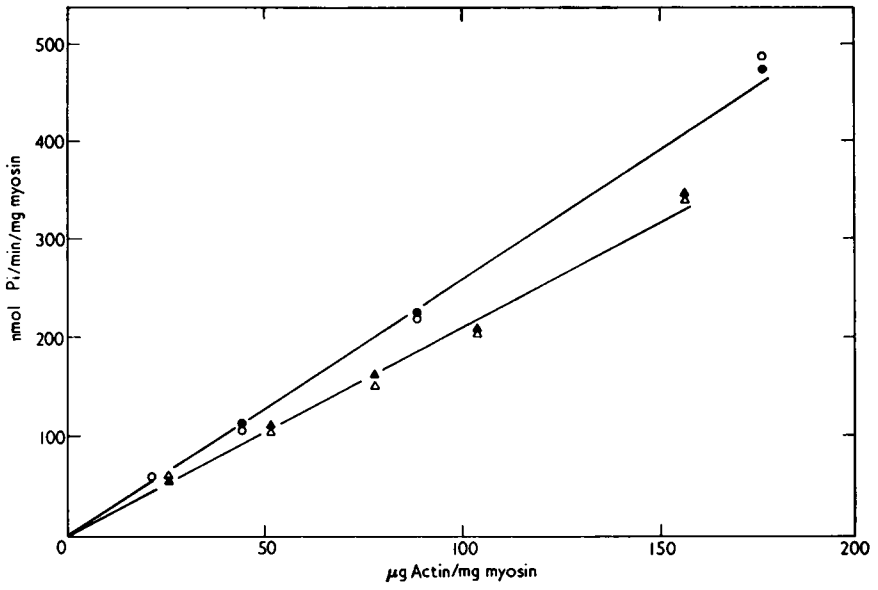

FIGURE 3 ATPase activation of rabbit myosin by pure actin and by thin filament preparations. ○——○, ●——●, rabbit actin; △——△, ▲——▲, thin filaments from red adductors of *Mercenaria mercenaria*. Conditions: 30 mM KCl, 1 mM $MgCl_2$, and 0.5 mM ATP at pH 7.6. Empty symbols: in the presence of 0.01 mM $CaCl_2$. Filled symbols: in the presence of 0.1 mM EGTA. (From Szent-Györgyi et al., 1971.)

Purified molluscan myosins differ from rabbit myosin and form a calcium regulated complex with pure rabbit actin. Molluscan myosins also bind calcium with a high affinity in the presence of a great excess of magnesium (Table I). Molluscan thin filaments consist mainly of actin and tropomyosin and, in contrast to thin filaments from rabbit muscle, do not bind calcium and are not regulated by it (Table II, Fig. 3) (Kendrick-Jones et al., 1970; Lehman et al., 1972; Lehman and Szent-Györgyi, 1975).

Myosin control operates with pure actin, free of troponin and tropomyosin. In this control system the properties of myosin are altered by calcium. Myosin control depends on the ability of particular myosins to respond to calcium in the physiological ranges of calcium and magnesium concentrations.

DISTRIBUTION OF THE REGULATORY SYSTEMS

In the animal kingdom some muscles contain regulated myosins, some contain regulated actins, and some muscles are doubly regulated.

The most direct way to distinguish between the two regulatory systems is to isolate thin filaments and myosins and see if they are calcium controlled by hybridizing them with pure rabbit proteins. Frequently, however, the muscles are too small for isolation of the pure proteins, or regulatory functions may be lost during the steps required for isolation (Lehman et al., 1974; Lehman and Szent-Györgyi, 1975). Since the mechanism of the two regulations is different, it is possible to probe for a particular regula-

TABLE I

COMPARISON OF MYOSINS

Source	% Inhibition of ATPase in absence of Ca^{++} with pure actin	μmol Ca/g 5×10^{-6} M Ca^{++}
Rabbit	0	< 0.3
Aequipecten (striated)	92	2.8
Aequipecten (smooth)	> 95	2.4
Mercenaria (red)	84	2.3
Glottidia (peduncle)	70	2.5

ATPase activity was measured in a pH-Stat by following proton liberation at pH 7.5 at 23°C. 0.5–2 mg myosin was mixed with rabbit actin in 0.4 M NaCl at a ratio of 2–3 g to 1 g actin. The rabbit actin used was free of tropomyosin and troponin contamination and moved as a single band in SDS-acrylamide gel electrophoresis. The actomyosin formed was stirred into 10 ml 25 mM NaCl (final concentration), 1 mM $MgCl_2$, 0.5 mM ATP which additionally contained either 0.1 mM $CaCl_2$ or 0.1 mM EGTA. Frequently calcium was added directly to the EGTA-containing sample once the ratio of proton liberation was established. Inhibition of ATPase activity equals (1 – [ATPase in EGTA/ATPase in Ca^{++}]) × 100. Calcium binding was measured as described by Kendrick-Jones et al. (1970).
(From Lehman et al., 1972.)

tion in crude muscle extracts. The effect of tropomyosin and troponin on actin is to reduce the affinity of actin for myosin. Thus, one may test for the presence of myosin-control by adding pure actin to a crude actomyosin extract in the absence of calcium. If the myosin is not regulated, it combines preferentially with the pure actin, and the ATPase activity of the actomyosin extract is activated by pure actin. If the actomyosin

TABLE II

COMPARISON OF THIN FILAMENTS

Source	% Inhibition of ATPase in absence of Ca^{++} with rabbit myosin	μmol Ca/g at 5×10^{-6} M Ca^{++}
Rabbit	50–93	~4.0
Chicken	82	~2.0
Frog	50	~2.0
Limulus	76–97	0.6
Lethocerus	78	0.8
Nereis	51	0.5
Glycera	62	0.5
Lumbricus	60	1.3
Aequipecten (striated)	< 3	< 0.1
Mercenaria (pink)	< 3	< 0.1
ABRM	< 3	—
Glottidia	0	—
Mercenaria and rabbit relaxing proteins	70	~6.0

Thin filament preparations were mixed with purified rabbit myosin in various proportions (1 g to 5–20 g). ATPase measurements and calcium binding were as described in the legend of Table I.
(From Lehman et al., 1972.)

preparation contains regulated myosin, the ATPase activity remains low with excess pure actin since regulated myosin is unable to combine with pure actin without calcium (Fig. 4). This competitive actin activation test probes for the presence of regulated myosin. If the myosin in the preparation has been found to be regulated, further tests are then required to establish whether or not a muscle contains, in addition, regulated actin. Muscle extracts may be assayed for actin-control with the aid of pure rabbit myosin. If regulated actin is present, the ATPase activity of the rabbit myosin becomes calcium sensitive; in the absence of actin control, this activity does not depend on calcium. The competitive myosin activation assay can only be used with muscles that have a relatively low ATPase activity because, if the background level of ATPase is high, it is difficult to evaluate the calcium sensitivity of the added rabbit myosin. The demonstration of the coexistence of actin control with myosin control frequently requires the isolation and the testing of thin filaments (Lehman et al., 1972; Lehman and Szent-Györgyi, 1975).

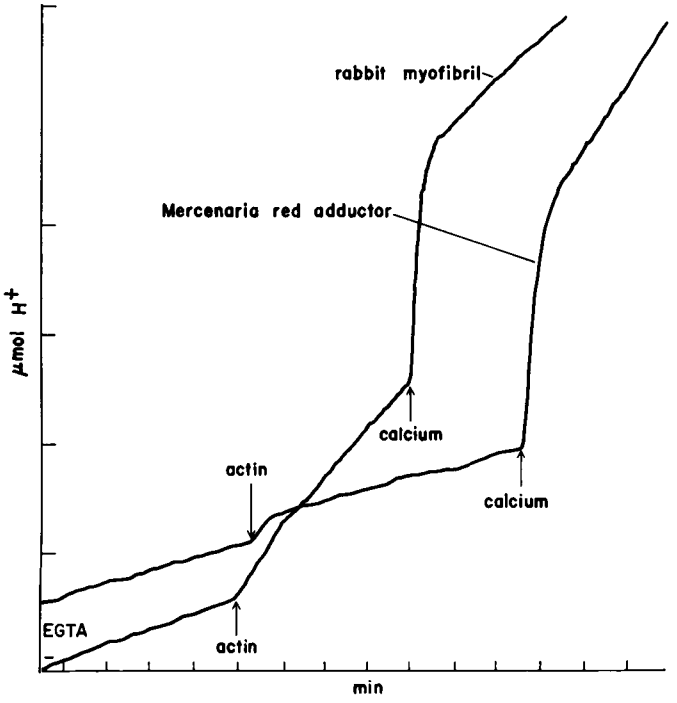

FIGURE 4 Response of myosin-linked and actin-linked regulatory systems to pure rabbit actin; pH-stat records of proton liberation. To 0.8 mg rabbit myofibrils and 1.5 mg *Mercenaria* muscle 0.4 and 0.7 mg rabbit actin were added (arrow). The ATPase of rabbit myofibrils is fully activated by actin, and no further increase is observed on the addition of calcium (arrow). In the case of *Mercenaria* myofibrils, actin has no effect; calcium is needed for activation. The burst of proton liberation upon addition of calcium is due to displacement of protons on the EGTA by calcium. 0.1 mM EGTA, 25 mM NaCl, 1 mM $MgCl_2$, and 0.5 mM ATP followed by 0.2 mM $CaCl_2$. (From Lehman et al., 1972.)

Both myosin control and actin control are widely distributed. Myosin-linked regulation has been demonstrated in the muscles of nematodes, nemertine worms, priapulids, echiuroids, sipunculids, brachiopods, molluscs, annelids, crustaceans, arachnids, insects, and echinoderms. Actin-linked regulation has been found in the muscles of nematodes, priapulids, sipunculids, crustaceans, annelids, arachnids, insects, and vertebrates (Fig. 5). Many muscles are doubly regulated; muscles with a single regulatory system are rather the exception. Myosin-control is not functioning in the striated muscles of vertebrates and in some crustacean muscles, such as the fast muscles of decapods and mysids. Actin control is not found in the muscles of molluscs, brachiopods, echinoderms, echiuroids, and nemertine worms (Lehman and Szent-Györgyi, 1975).

In some animals different groups of muscles may differ in regulation. The fast tail and deep abdominal extensor muscles of lobster showed only actin control. In the slow crusher claw muscles and the superficial abdominal extensor muscles of lobster both regulations were functioning (Lehman and Szent-Györgyi, 1975). The slow and fast lobster muscles differ also in sarcomere and thick filament lengths (Jahromi and Atwood, 1971), paramyosin content, and ATPase activity. Myosin-linked regulation was also found in chicken gizzard muscles (Bremel, 1974). Actin control has been reported in slime mold (Nachmias and Asch, 1974).

COMPONENTS OF MYOSIN-LINKED REGULATION

In myosin-linked regulation myosin sites involved in combination with actin become available in the presence of calcium. The question arises as to whether or not regulatory components of myosin can be identified and how such components modify the properties of myosin. This problem has been studied on the myosin of striated adductor muscles of scallop. There is evidence, however, that the major conclusions apply to other myosin-linked regulatory systems.

The subunit structure, size, and many of the properties of scallop myosin are similar to vertebrate myosins. Scallop myosin consists of two heavy chains of nearly 200,000 daltons and of four light chains of about 18,000 daltons. The heavy chains form an α-helical rod-like region at the tail end of the molecule and two globular head regions containing the ATPase and actin-combining centers. Proteolytic fragments, such as subfragment-1 (S-1), myosin rods and meromyosins similar in size and properties to those obtained from rabbit, can be readily prepared. Although they are of similar size, there are two kinds of light chains; one of each is probably associated with a subfragment-1 (Szent-Györgyi et al., 1973).

Scallop myofibrils and myosin preparations lose their calcium sensitivity when exposed to 1-10 mM EDTA solutions. EDTA detaches a mole of light chain from myosin (Kendrick-Jones et al., 1972; Szent-Györgyi et al., 1973). The residual myosin is a "desensitized" myosin since its ATPase activity does not depend on calcium and is no longer inhibited in the absence of calcium. The calcium binding of the "desensitized" myosin is reduced to about half of the value of untreated myosins. The isolated light

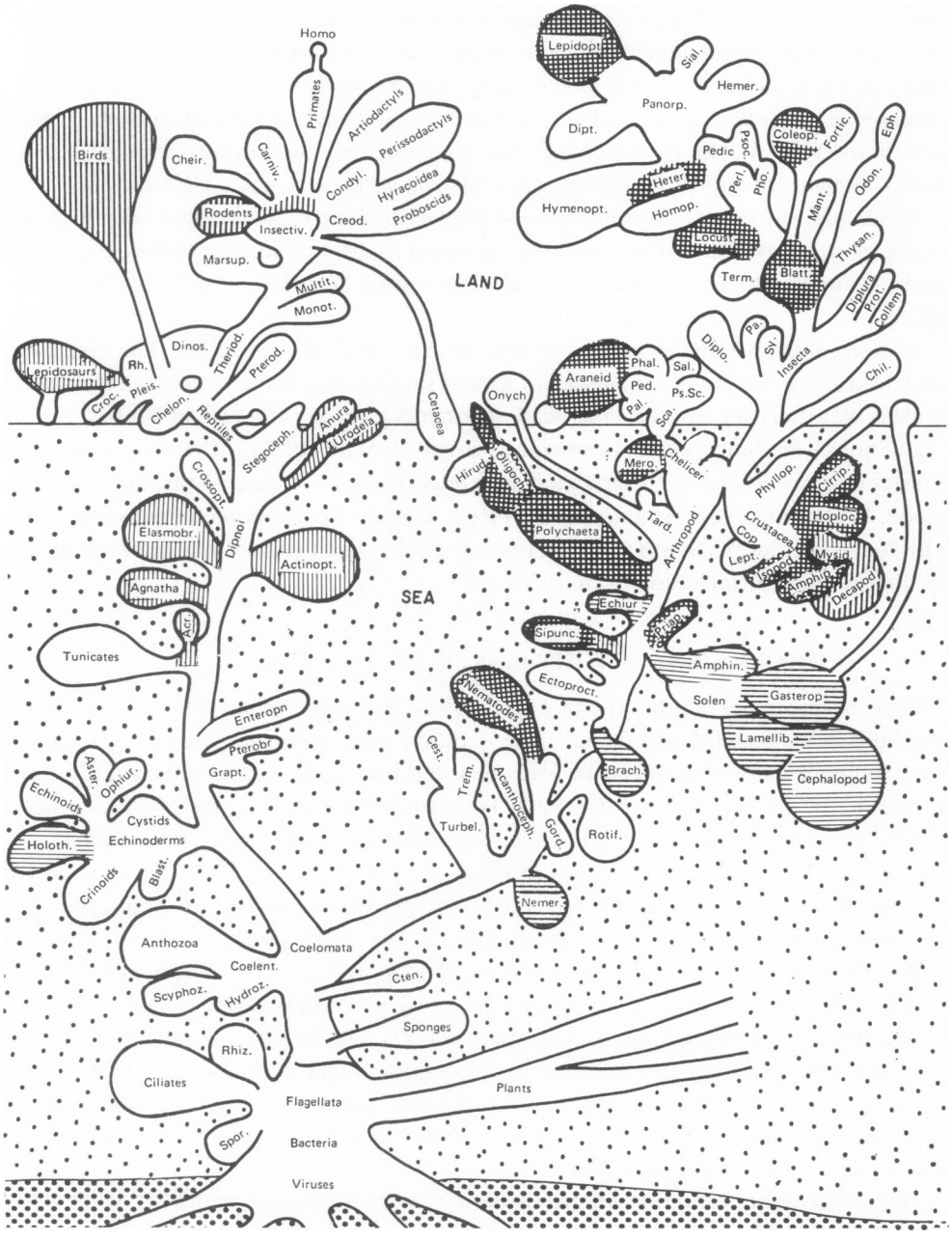

FIGURE 5 Distribution of regulatory systems in the animal kingdom. Vertical lines: presence of actin-control; horizontal lines: presence of myosin-control. Note that both controls are present in many invertebrate muscles. Evolutionary tree from Borradaile et al. (1963). (From Lehman and Szent-Györgyi, 1975.)

chain has no ATPase activity and it does not bind calcium (Table III). In the presence of magnesium ions the light chain recombines with stripped scallop myosin. The recombined myosin regains calcium sensitivity and its calcium binding is like that of untreated preparations (Table III). The dependence of myosin-control on the presence of the light chain released by EDTA, indicates the direct involvement of this particular light chain in regulation and suggests that the light chain is a regulatory subunit of scallop myosin (Szent-Györgyi et al., 1973).

Although it has turned out that there are two regulatory light chains in a myosin molecule (Kendrick-Jones, Szentkiralyi, and Szent-Györgyi, to be published), removal of only one by EDTA desensitizes the myosin or the myofibrils fully. The stripped myosin preparation from which a regulatory light chain has been removed by EDTA recombines with no more than one light chain. These experiments indicate that two light chains are required to regulate myosin and that the prevention of complex formation with actin in the absence of calcium, the "off" state, is the result of a joint action of both regulatory light chains. The results, however, do not imply a cooperativity between the two heads during the ATPase activity in the presence of calcium. It is of interest that the proteolytic fragment, heavy meromyosin, which contains the two myosin heads connected with a short tail portion, retains calcium sensitivity. In contrast, S-1 preparations, consisting of single myosin heads are not calcium sensitive, although they bind calcium and contain the light chains. S-1 preparations are not regulated even in the presence of excess regulatory light chains obtained from intact myosin that was not exposed to papain digestion (Szent-Györgyi et al., 1973). It ap-

TABLE III

EFFECT OF REGULATORY LIGHT CHAIN ON ATPASE ACTIVITY AND ON CALCIUM BINDING OF SCALLOP MYOSIN

	Actin-activated ATPase*		Ratio EGTA/Ca^{++}	Calcium binding‡
	0.1 mM Ca^{++}	0.1 mM EGTA		
Aequipecten				
Untreated myosin	0.22	0.05	0.23	1.67
"Desensitized" myosin	0.24	0.20	0.83	0.78
"Desensitized" myosin recombined with EDTA light chain in original proportions	0.22	0.07	0.32	1.59
EDTA light chain	None	None		None

*Values are given as micromoles ATP per milligram per minute at 23°.

‡Values are in nanomoles Ca^{++} per milligram protein at 5×10^{-7} M Ca^{++}.

Myosin was "desensitized" by 10 min treatment with 10 mM EDTA in 40 mM NaCl and 10 mM pH 7.0 phosphate at 0°. The myosin was centrifuged, washed twice with 40 mM NaCl, 10 mM phosphate, pH 7.0, and redissolved in 0.6 M NaCl, 5 mM phosphate pH 7.0. The "desensitized" myosin was recombined with the EDTA-light chain by adding 10 mM $MgCl_2$ to the supernatant liquid obtained after EDTA treatment. The pH was readjusted to 7.0 and the light chain fraction and desensitized myosin were mixed in 0.6 M NaCl in the original proportions. The mixture was dialyzed against 40 mM NaCl, 1 mM $MgCl_2$, and 5 mM phosphate pH 7.0 overnight and was washed with the same solution.

(From Kendrick-Jones et al., 1972.)

pears that removal of a single regulatory light chain influences both heads of myosin. An alternative explanation would have to assume that there are drastic differences between the two myosin heads, only one head having an ATPase activity. This is an unlikely possibility.

The regulatory light chain remaining on scallop myosin after EDTA treatment is removed by dithiodinitrobenzoic acid (DTNB). DTNB treatment, however, results in an irreversible loss of ATPase activity. The regulatory light chains obtained by EDTA and by DTNB are identical on SDS and urea acrylamide gels, are indistinguishable in composition or on tryptic peptide maps, have the same ultraviolet spectra and resensitize EDTA stripped myofibrils at the same light chain to myosin ratio (Fig. 6). Little is known of the function of the light chains that remain on myosin after the removal of the two regulatory light chains. These light chains are obtained by guanidine-HCl treatment of a myosin from which both regulatory light chains had been previously removed by EDTA and by DTNB. There are two such guanidine light chains on scallop myosin. They differ chemically from the regulatory light chains, do not combine with a desensitized myosin preparation, and do not effect its ATPase activity (Fig. 6) (Kendrick-Jones, Szentkiralyi, and Szent-Györgyi, to be published).

Although scallop myosin or myofibrils are readily desensitized by EDTA, response to EDTA treatment is not a good indicator for myosin-linked regulation. In fact, scallop is the only muscle we have encountered that is fully desensitized by EDTA, and it was rather fortunate that it was the first system chosen to be analyzed. EDTA may have only a partial or no effect at all on other molluscan muscles, such as *Mercenaria* or squid, although regulatory light chains can also be obtained from these muscles by procedures that result in an irreversible loss of ATPase activity. These regulatory light chains differ in amino acid composition and in tryptic peptide from the regulatory light chains of scallop. Nevertheless, they hybridize with desensitized scallop myofibrils in a mole to mole ratio and restore calcium sensitivity fully. Even more striking is the discovery of Kendrick-Jones (1974) that the DTNB light chain of rabbit hybridizes with a desensitized scallop myofibril and restores regulatory function. This observation indicates that the lack of myosin control in rabbit muscles is not due to a lack of regulatory light chain in myosin. Recently, regulatory light chains, i.e. light chains that hybridize with desensitized scallop myosins and confer calcium sensitivity, have been obtained from other muscles that appear to lack a functioning myosin control, including frog, beef heart, lobster muscles (Kendrick-Jones, Szentkiralyi, and Szent-Györgyi, to be published).

SPECULATIONS

Although scallop myosin is the only available one for hybridization studies since other myosins cannot be desensitized without loss of ATPase activity, it has been very useful for comparative studies. The surprising ease of hybrid formation between the regulatory light chains of various organisms and stripped scallop myosin indicates that certain regions of the heavy and light chains have been conserved. The explanation for

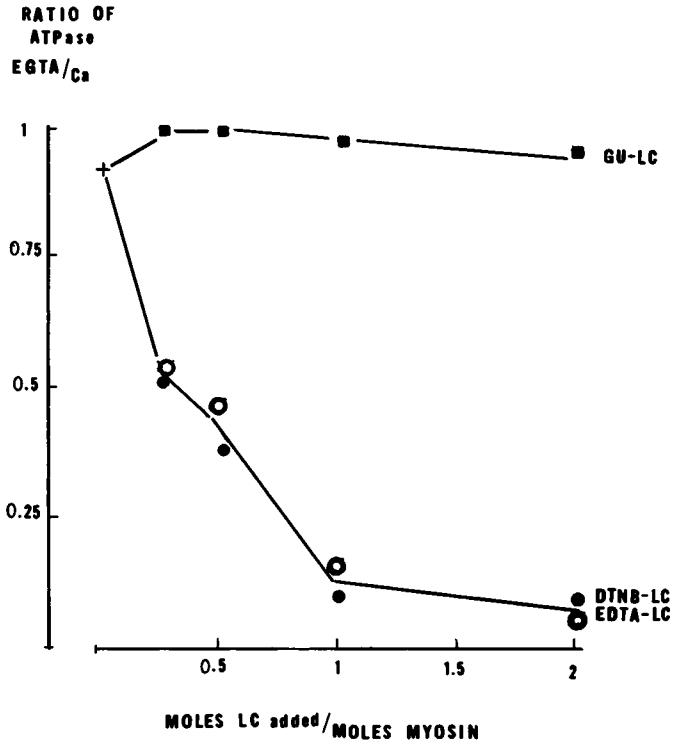

FIGURE 6 Resensitization of scallop myofibrils by different light chain fractions. Scallop myofibrils were desensitized with 10 mM EDTA for 10 min at 0° in 40 mM NaCl, 5 mM phosphate pH 7.0, washed twice with 40 mM NaCl, 5 mM phosphate pH 7.0 and resuspended in 40 mM NaCl, 5 mM phosphate pH 7.0, 1 mM $MgCl_2$. Myofibrils at 2 mg/ml protein concentrations were incubated with light chain fractions of various concentrations, shown on abscissa for overnight with gentle stirring, centrifuged, and washed twice in 40 mM NaCl, 5 mM phosphate pH 7.0, 1 mM $MgCl_2$. Myosin content of myofibrils was taken 60% (Szent-Györgyi et al., 1973). Light chains were prepared from myofibrils sequentially and purified on DEAE-Biogel-A at pH 6.0 on phosphate gradients 25–400 mM and on Sephadex G-100 columns. The light chains showed a single band on SDS-acrylamide and urea-acrylamide gel chromatography. EDTA-LC (○) was prepared at first. DTNB-LC (●) was obtained from the muscle residue by 10 min DTNB (10 mM) treatment at pH 8.1, followed by centrifugation at pH 6.5. The supernatant was dialyzed extensively against 0.1 mM DTT, 40 mM NaCl, 5 mM phosphate, pH 7.0. The muscle residue was washed twice and treated overnight with 6 M guanidine-HCl in the presence of 10 mM dithiothreitol at 0°. The solution was diluted with $2\frac{1}{2}$ vol of water and 3 vol of ethanol. The precipitate was removed and the supernate was concentrated in a flash evaporator, dialyzed against 0.1 mM dithiothreitol, 40 mM NaCl, 5 mM phosphate, pH 7.0. The Gu-LC (■) was purified by DEAE and Sephadex column chromatography. (Kendrick-Jones, Szentkiralyi and Szent-Györgyi, to be published.)

such an invariance probably lies in the great similarity of actin sequences throughout evolution (Elzinga et al., 1973). It is likely that the portion of myosin that combines with actin, therefore, also had to retain constant features during evolution. Such rationalization positions the regulatory light chains near the actin-combining sites of myosin and would support a speculation that the light chains sterically block sites on

myosin in the "off" state, and may move out of the way during the "on" state. Such a speculation provides a model which can also be subjected to experimental tests.

All the components of myosin regulation are associated with myosin while all the components of actin control are associated with the thin filaments. Regulatory light chains do not bind to the thin filaments and troponin or tropomyosin do not combine with myosin. A common evolutionary origin for troponin-C, myosin light chains and parvalbumin has been proposed recently on the basis of homologous amino acid sequences (Collins, 1974; Weeds and McLachlan, 1974; Tufty and Kretsinger, 1975). However, no functional relation can be demonstrated between troponin-C and the regulatory light chains. The two kinds of regulations appear to act independently of each other, although their effect may be additive.

There is no simple relationship between a particular regulatory system and the physiological performance of the muscle or its structure. Double regulation appears to be advantageous, since reliable control over rest and activity may be easier to maintain. It is difficult to see the advantages of single systems. Since regulation can be lost as a result of experimental manipulations, evidence for single systems deserve careful attention. The lack of actin control in molluscan muscles is supported by the finding that these muscles are deficient in troponin (Lehman et al., 1972; Lehman and Szent-Györgyi, 1975). Lack of myosin control in vertebrate striated muscles, however, is not due to lack of regulatory light chains. The disappearance of the ordered crossbridge lattice upon stimulation in frog muscles that have been stretched beyond overlap (Haselgrove, 1972, 1975) and the calcium dependence of the viscosity and sedimentation properties of isolated and reconstituted thick filaments (Morimoto and Harrington, 1974) indicate that calcium may interact directly with vertebrate myosin at physiological concentrations. These observations, however, do not directly demonstrate a myosin control in vertebrate striated muscles. Myosins of vertebrate striated muscles when complexed with pure actin show no calcium sensitivity. Although it is difficult to exclude in vivo myosin control on the basis of in vitro evidence, it is quite possible that the lack of myosin control in vertebrate striated muscles is due to mutations in the heavy chains of myosin that restrict their interactions with the regulatory light chains.

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