Calcium Activation of Fibers

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Introduction

The function of muscle is to contract. Contraction occurs through the sliding of two sets of filaments when the two major contractile proteins, myosin and actin, interact in a cyclic fashion. The energy driving this mechanochemical event is derived from ATP and the regulation of the cyclic interaction depends upon the presence of calcium. These features of contraction are common to all muscle types, skeletal, cardiac and smooth, within the same species, and in the muscles of different species. However, the regulatory mechanisms of the actin•myosin•ATP interaction are quite different. But the point of regulation, the interaction of myosin and actin and the proteins which bind calcium are very similar to all muscle types.

Activation of the actin•myosin•ATP interaction within a skeletal muscle cell by calcium is preceded by several carefully regulated events. A muscle fiber action potential occurs when an action potential of a motor neuron is transmitted to a muscle fiber via the neuromuscular junction. The action potential of the muscle fiber leads to a transient elevation of intracellular calcium from a resting level of 10^{-7} M to an activating level of 10^{-5} M when calcium stores are released from the terminal cisternae. Calcium binding to the thin filament, as is the case in striated muscle, allows for the transformation of chemical energy, through the interaction of myosin with actin, into the usable mechanical forms of muscle force and/or shortening. Relaxation occurs when the myoplasmic calcium is lowered to resting levels by the sarcoplasmic reticulum followed by calcium removal from the contractile component.

Unraveling the role of calcium in muscle contraction began in 1883 when Sydney Ringer noted that frog hearts continued to beat only when bathed in solutions made with London tap water and that water from other sources would not do. The critical ingredient was found to be dissolved calcium. It was not until over a half a century later that the role of calcium in muscle contraction would be elucidated as we know it today.

Contaminating levels of calcium in water, ATP and other chemicals used in muscle studies were more than adequate to elicit contraction, and no doubt, hampered studies on the molecular mechanism of regulation. Removal of calcium would clearly enhance the observation of regulation and provide a cornerstone to our understanding of a regulatory mechanism. A factor from muscle homogenates was found by Marsh (1952) to relax myofibrils previously contracted with ATP. Several years later, Ebashi (1960) showed that the relaxing factor found by Marsh (1952), the sarcoplasmic reticulum, acted by removing calcium from actomyosin. Ebashi and his colleagues (1968) later showed that the proteins troponin and tropomyosin regulated the interaction of myosin and actin through calcium. Further, troponin was found to consist of three subunits with quite distinct properties, troponin-T, troponin-I, and troponin-C, and that the latter subunit bound calcium (Greaser and Gergely, 1973). A considerable amount of work has since been carried out on the biochemical properties of these regulatory proteins and the physiological role they play in the regulation of muscle contraction.

The purpose of this article is: First, to review the essential features of force and shortening when a muscle is activated; Second, to review current models for the interaction of myosin and actin, and the regulation of this interaction by calcium; Finally, to look at calcium activation of muscle fibers and the factors which modulate the response. Further background information may be obtained from several excellent reviews (Squire, 1981; Bagshaw, 1982; Peachy et al., 1983; Sheterline, 1983).

Physiology of Force and Velocity

Physiological States

Muscles can exist in three qualitative physiological states: relaxed, activated and rigor. In the relaxed state, the muscle is quite extensible. When activated, the muscle can shorten, lift loads and do work. If the metabolic affairs of the cell cease so that ATP is not replenished, then the muscle becomes inextensible; the condition known as rigor. If the sarcolemma of a muscle fiber was removed so that only the contractile network was left, a skinned fiber, then the above states may be demonstrated by bathing this fiber in solutions which mimic the intracellular condition of the state desired. However, skinned fibers may be manipulated to exist in four states; the three states described above but with the partitionment of the rigor state into two additional states. Calcium may be easily added to the rigor state in the skinned fiber by simply changing the solution that bathes it. Thus, there is a rigor-calcium state and a rigor-no calcium state. Furthermore, the advantage of a skinned fiber over an intact muscle fiber is the ability to reversibly change states easily. These states describe the interaction of actin and myosin, and the limits of regulation.

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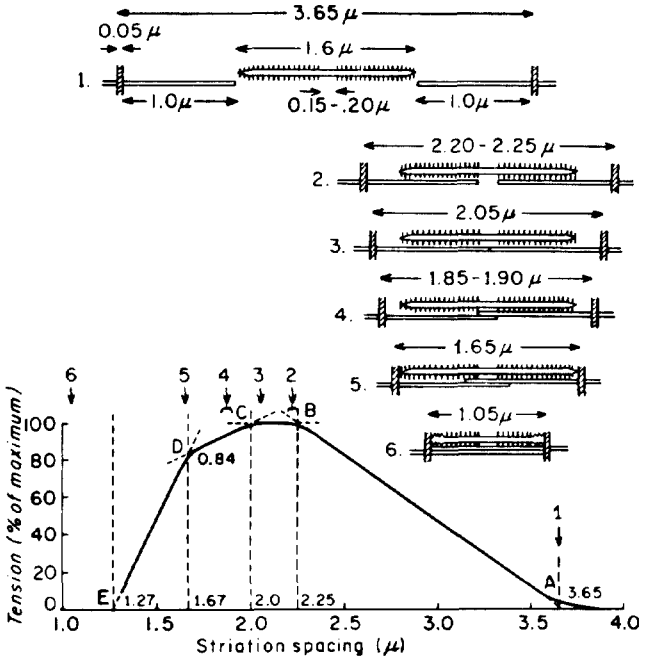
Force-Length Relationship

The dependence of force on muscle length has been recognized for over a century. When the muscle length is held constant, the force developed by a muscle is termed isometric. The amount of force at rest length, usually the in situ length, is maximal while with longer or shorter lengths, the force declines. This relationship was quantitated in the classic length-tension studies of Gordon et al. (1966) which provided strong evidence for the in vivo interaction of thick and thin filaments in force production. The postulation by Huxley (1957) of independent force generators on the thick filament was a cornerstone of the sliding filament theory of contraction. If indeed the myosin cross-bridges were the site of interaction, then as the length of a muscle increased, the overlap between filaments would decrease and so would the number of cross-bridges capable of interacting with the thin filament. Their results (Gordon et al., 1966) and a diagrammatic correlation with the structural information of Page and Huxley (1963), Figure 1, show the key points of the force-length relationship. Beyond overlap (A and greater, Fig. 1) there can be no interaction so the force is zero, but increases in overlap accompany increases in force (traveling from A → B, the descending limb of the force-length curve, Fig. 1). The middle of the thick filament lacks cross-bridges (pseudo H-zone) and as such, shortening of the sarcomere from 2.2 to 2.0 μm (traveling from B → C) provides no additional opportunity for bridge formation and force remains constant in this region. At even shorter sarcomere lengths (traveling from C → D → E, termed the ascending limb, Fig. 1), force drops again, perhaps related to the mechanical collision or overlap of the thin filaments and finally, collision of the thick filaments with the Z-line. The structural and physiological complementary results strongly support the hypothesis that cross-bridges are the site of interaction between thick and thin filaments.

Figure 1



The relationship between the force-length curve and sarcomere length for single muscle fibers. The numbers to the left of each filament diagram at different sarcomere lengths corresponds with the numbers marked with arrows on the force-length relationship below. A, Active force is zero at this or longer sarcomere lengths. B, The beginning of the plateau phase of force. C, The shorter sarcomere length limit of the plateau. D, The sarcomere length at which the slope becomes steeper. E, The short sarcomere length limit at which active force is also zero. (After Gordon et al., 1966. J. Physiol. (Lond.) 184:170)

Force-Velocity Relationship

The force that a muscle generates will depend on its length (as discussed in the force-length relationship above) and speed of shortening. If the load attached is less than the isometric force, then sustained shortening occurs with the force developed by the muscle being equal to the load being lifted (see Fig. 2a). As the load attached to the muscle decreases, the speed of shortening or velocity will increase as shown in Figure 2, and as the load increases, velocity decreases. This hyperbolic relationship (Fig. 2b) is referred to as the force-velocity relationship. Maximum velocity is obtained when the muscle contracts against a zero load, in which case the speed of shortening depends more on muscle type and correlates with the V_{max} of the myosin ATPase. A.V. Hill (1938) described the energetics and a postulated mechanism, the interaction of points, or cross-bridges as they are presently referred to, of this relationship in his eloquent and classic paper on muscle energetics.

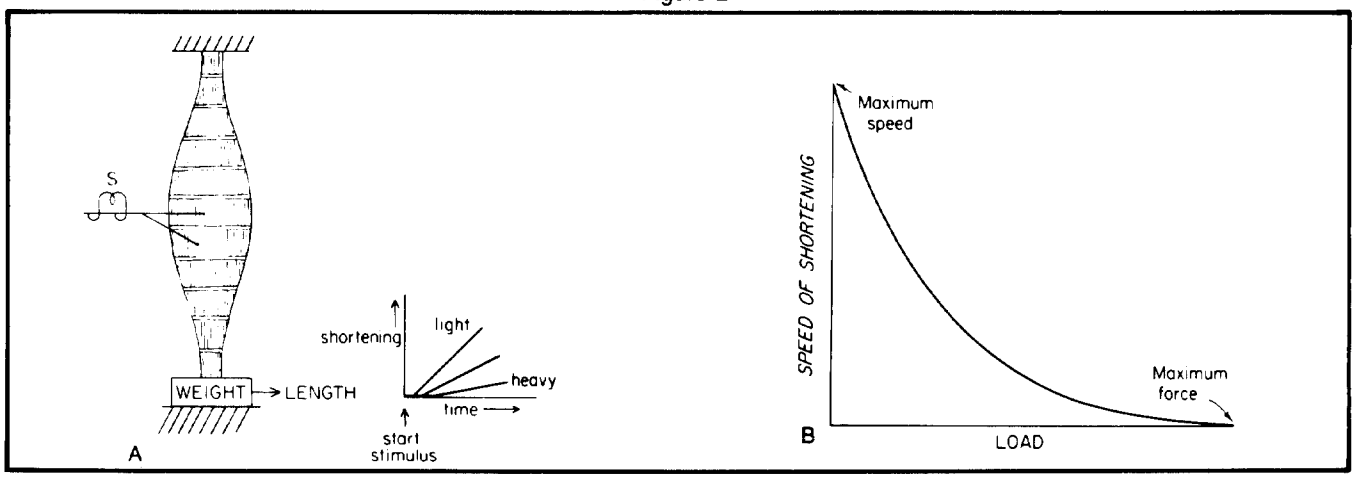
> "Imagine that the chemical transformations associated with the state of activity in muscle occur by combination at, or by the catalytic effect of, or perhaps by passage through, certain active points in the molecular machinery, the number of which is determined by the tension existing in the muscle at that moment. We can imagine that when the force in the muscle is high the affinities of more of these points are being satisfied by the attractions they exert on one another, and that fewer of them are available to take part in chemical transformation. When the tension is low the affinities of less of these points are being satisfied by the mutual attraction, and more of them are exposed to chemical reaction."

Thus, when the load is great, there are more attached cross-bridges and the force is great. And conversely, when the load is light, the cross-bridges are free to cycle and the velocity becomes greater.

Regulation of the Actin•Myosin•ATP Interaction

The ability of skeletal muscle to contract depends on calcium binding to the regulatory protein troponin, and the interaction of the myosin•nucleotide complex with actin for force and/or shortening with energy release. Understanding the molecular basis for this mechanochemical event is essential for the elucidation and understanding of the regulation of muscle contraction by calcium.

Figure 2



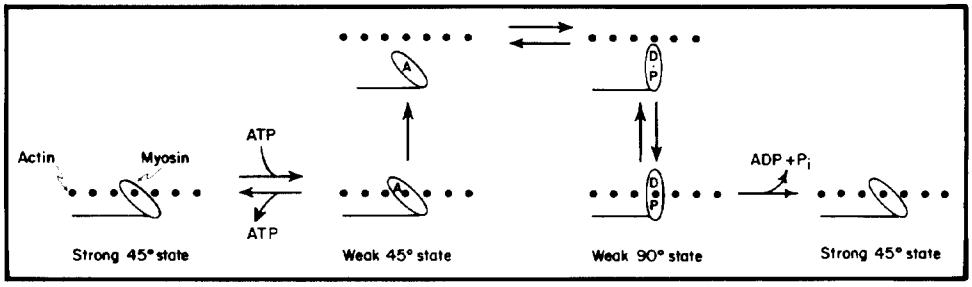
The relationship between muscle shortening velocity and force. A, Diagram of an experiment to measure muscle shortening when the muscle is tetanically stimulated. The mass is not lifted until the muscle force equals the load. Muscle length is plotted against time for three loads. The slope of each curve is the shortening velocity. B, Plot of data from A showing the inverse relationship between velocity and load.

Actin•Myosin•ATP Interaction

A key step to the understanding of muscle contraction resulted from the cross-bridge model of Lymn and Taylor (1971). Two important observations became the basis for their model. One, myosin hydrolyzes ATP much more rapidly than the subsequent release of products, ADP and P_i, from the active site (Fig. 3) (Lymn and Taylor, 1970). And two, ATP decreased the affinity of myosin for actin and further that the dissociation of actin•myosin by ATP was found to occur more rapidly than the hydrolysis of ATP by myosin (Lymn and Taylor, 1971). With these results a cross-bridge model was formulated (Fig. 3) on premises that myosin must detach from actin before ATP is hydrolyzed and that the dissociation of actin•myosin by ATP appeared to be irreversible, assumptions that were later challenged. Furthermore, each kinetic step was structurally correlated with an in vivo structural change. Thus, myosin, in a myosin•actin rigor or schematic 45^0 state, is dissociated from actin by the strong binding of ATP to the cross-bridge (myosin•actin + ATP → myosin•ATP + actin). Myosin then hydrolyzes ATP (myosin•ATP ↔ myosin•ADP•P_i) while retaining the hydrolysis products and the cross-bridge angle returns to the schematic 90° state (Fig. 3). Myosin•ADP-P_i then reattaches to actin for the energy-releasing stroke and release of products. This model was consistent with earlier models of cycling cross-bridges and provided a valuable framework for investigating the mechanochemical coupling between ATP and contraction.

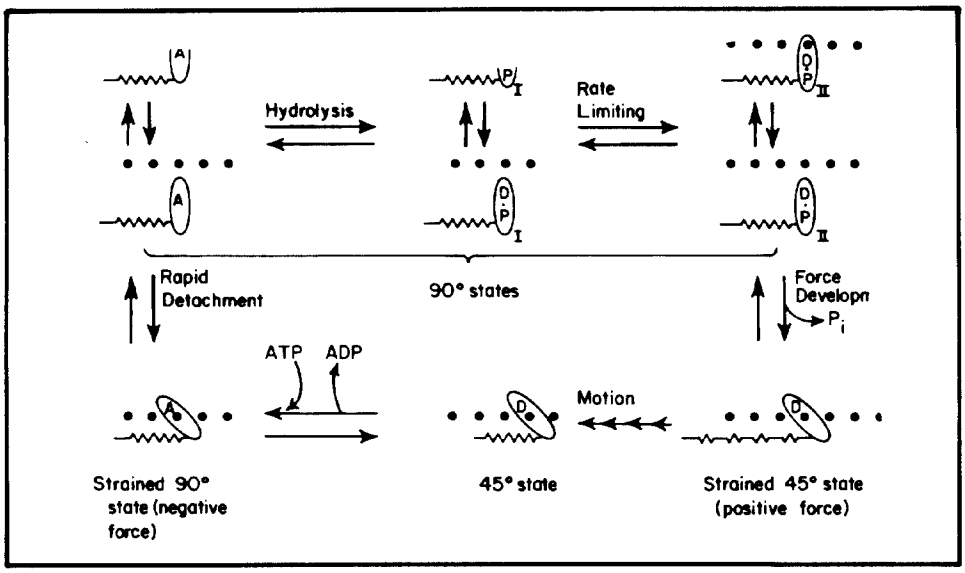
However, the key assumptions of the Lymn-Taylor cross-bridge model, that is, the irreversibility of the dissociation of myosin from actin and the hydrolysis of ATP by myosin after this dissociation, were later challenged. Myosin was found to hydrolyze ATP while bound to actin (Mornet et al., 1981; Stein et al., 1981). Furthermore, myosin•ATP existed in rapid equilibrium with actin•myosin•ATP, as well as the other intermediate forms being in equilibrium with the appropriate myosin•nucleotide intermediates (Stein et al., 1979). Thus, all of the myosin states reversibly bind actin. In addition, myosin•ATP and myosin•ADP•P_i^n (where n represents intermediates of this form) were found to bind weakly while myosin and myosin•ADP have a much greater affinity for actin. These results implied that the presence of the terminal or γ-phosphate of ATP on myosin determines the weak binding state and its release transforms myosin into the strong binding state.

Figure 3



The Lymn and Taylor cross-bridge model of muscle contraction. The lower portion of the diagram represents bound cross-bridges. The upper portion represents detached cross-bridges. Bound ATP is symbolized by the letter A, ADP by the letter D and phosphate by the letter P. (Adapted from Lymn and Taylor, 1971. Biochem. 10:4617)

Figure 4



The Eisenberg and Greene cross-bridge model of muscle contraction. Symbols and abbreviations are the same as those in Figure 3. The upper portion of the diagram, labeled 90° states, represents the rapid equilibrium or weak binding states of the cross-bridge for the thin filament. The lower portion depicts the strong binding states. The multi-arrowed line represents a continuous conformational change of the cross-bridge which occur during filament sliding. (Adapted from Eisenberg and Greene, 1980. Ann. Rev. Physiol. 42:293; and Eisenberg and Hill, 1985. Science 227:999)

Based on these additional observations, Eisenberg and Greene (1980) formulated a cross-bridge model (Fig. 4) that also contained many of the essential features of the physiological model of Huxley and Simmons (1971). Furthermore, they assumed that in product release, P_i release is essentially an all or none process and that ADP release occurs following the work stroke. The differences in binding of the weak state and the strong state were proposed to reflect a structural difference in binding and that the rate constants associated with energy transduction were sensitive to the mechanical strain of the cross-bridge. The major points of this model are shown in Figure 4. The cross-bridges are elastic structures and as such, may bind to the thin filament with a wide range of angles (Huxley and Simmons, 1971). In addition, there are preferred orientations of the cross-bridge which depend on the presence of the γ-phosphate of ATP. Consequently, the strong binding state would have a preferred orientation of 45^0 (actin•myosin•ADP and actin•myosin) and the weak binding state would have a preferred orientation of 90^0 (myosin•ATP, myosin•ADP•P_i, actin•myosin•ATP and actin•myosin•P_i). Energy transduction occurs with the release of products in a two-stage manner; release of P_i followed by the sequential release of ADP. Cross-bridges are then detached through the binding of ATP to actin•myosin but detachment does not necessarily occur as ATP is hydrolyzed, such as is postulated to occur during an isometric contraction.

The hydrolysis of ATP by myosin involves several steps or stages. Each stage during hydrolysis exists in rapid equilibrium between free and bound (upper portion of Fig. 4). Furthermore, the rate-limiting step of the myosin ATPase is much quicker than the rate-limiting step for energy transduction. That is, ATP is broken down faster than a cross-bridge can rotate. Upon activation, myosin•ADP•Pi binds to actin with the subsequent release of P_i. Associated with Pi release is an increased propensity for myosin to exist in a 45^0 state. The work stroke is completed with ADP release. Cross-bridges in the strained state would still contain bound ADP so ATP binding and subsequent hydrolysis would be precluded until the strain were relieved or until release of ADP. Thus a simple mechanism is conserved for cross-bridge energy cycling in which a cross-bridge with a low energy level is reverted to a cross-bridge possessing a different conformation and potential to do work (much higher free energy level) at the beginning of the cycle. During isotonic contractions, the cross-bridges in the actin•myosin•ATP state would rapidly detach from the thin filament. While in an isometric contraction, cross-bridges would not necessarily detach during ATP hydrolysis but would spend most of the time exerting force in the actin•myosin•ADP strained state or oscillate between the various attached states. It is interesting to note that this description is reminiscent of the statement of Hill (1938) (see force-velocity relationship) who described the interaction of points in the force-velocity relationship.

Based on accumulating evidence from both biochemical and physiological studies, there is general agreement on the essential features of the Eisenberg and Greene (1980) model. The most radical departure from the previous model is the existence of a weak binding 90^0 state of myosin to the thin filament in relaxed muscle. Biochemical studies show that both the weak and strong binding states decreased as the ionic strength increased and that the association constant is quite low at physiological ionic strength (Chalovich and Eisenberg, 1982; Greene et al., 1983). Stiffness measurements, a measure of cross-bridge attachment, and X-ray diffraction studies substantiated the biochemical observations (Brenner et al., 1984; Reiser and Moss, 1985). During activation, structurally different cross-bridges were found which appeared to be intermediate to rigor and relaxed

states of the cross-bridge (Burghardt et al., 1983; Matsuda and Podolsky, 1984; Brenner et al., 1984). Electron microscopy of crosslinked actin•myosin S-1 in the presence of ATP has recently revealed a variable angle of attachment between 90^0 and 45^0 states (Craig et al., 1985). This study further showed that different conformational states were associated with bound ATP and ADP. These studies provide evidence for the strained states of a cross-bridge in an isometric contraction and furthermore suggest that a weak binding state precedes the strong binding state. Since the presence and absence of the γ-phosphate on myosin appears to determine both the orientation and binding state of the cross-bridge, it would appear obvious, then, that calcium regulation would control this conversion.

Regulation of the Actin•Myosin•ATP Interaction

There appears to be three predominant mechanisms of regulation of muscle contraction: calcium interacting with troponin-C on the thin filament; myosin light chain-2 of myosin in the thick filament; or calmodulin for the activation of a kinase to phosphorylate a regulatory light chain of myosin. The proteins that calcium interacts with all share similarities in amino acid sequence and other important parameters (Kretsinger, 1980). The point of regulation for the three mechanisms is the same in that, upon an increase in calcium, myosin interacts with actin. However, only striated muscle regulation will be discussed (see Adelstein and Eisenberg, 1980; and Sheterline, 1983 for excellent reviews of regulatory mechanisms).

Calcium Activation of the Thin Filament

The regulatory complex of the thin filament consists of four proteins: troponin-T, troponin-I, troponin-C and tropomyosin. Troponin-T binds to tropomyosin and troponin-C though the latter is less stable in the presence of calcium. Troponin-I interacts with troponin-T, actin and troponin-C with the latter forming a stable complex in the presence of calcium. The addition of tropomyosin, troponin-T, and troponin-I to a mixture of myosin and actin inhibits the ATPase activity regardless of the calcium concentration. When troponin-C is included, the inhibition is relieved only in the presence of calcium.

Models of Calcium Activation

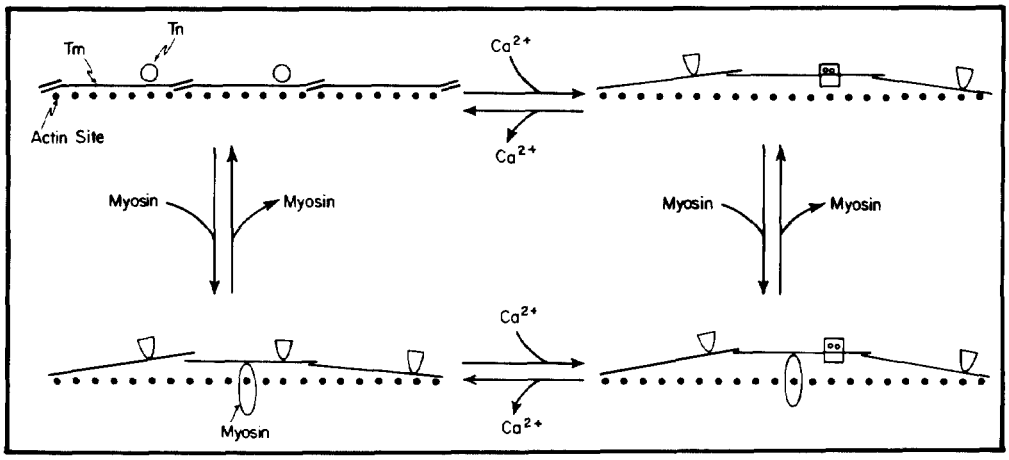
The steric blocking model was proposed as the major mechanism for the calcium control of skeletal muscle contraction (Haselgrove and Huxley, 1973; Parry and Squire, 1973). At rest, tropomyosin is in a position to physically block the interaction of myosin with actin. Calcium binding to troponin allows tropomyosin to move deeper into the groove of the actin double helix uncovering the myosin binding sites, thereby allowing myosin to interact with actin. Thus, in the absence of calcium, troponin keeps tropomyosin in a blocking position, while, following calcium binding to troponin, troponin allows tropomyosin to unblock the myosin binding sites on actin. This simple model of the control of muscle contraction, based on x-ray diffraction studies, provided a means of regulating contraction through a simple switch mechanism: calcium on, contraction occurs; calcium off, relaxation occurs.

Troponin-C has two low-affinity calcium-specific sites which bind calcium only (5×10^5 M^{-1}) and two calcium-magnesium sites which bind calcium with high affinity (2×10^7 M^{-1}) and, in addition, bind magnesium (Potter and Gergely, 1975). Work by Potter and Gergely (1975) led to the conclusion that calcium binding to the calcium-specific sites of troponin regulated the ATPase activity of myofibrils. Furthermore, calcium binding to the calcium-magnesium sites did not influence the calcium sensitivity of the myofibrillar ATPase or appear to influence calcium binding to the calcium-specific sites. On the basis of on and off rates for calcium binding to both classes of sites on troponin-C (Johnson et al., 1979), Robertson et al. (1981) calculated that in the presence of millimolar magnesium, as would occur in muscle, only calcium binding and removal from the calcium-specific sites could be rapid enough to account for the presumed calcium binding kinetics in intact fibers. This implied that the calcium-specific sites behaved as a simple calcium binding switch, a mechanism consistent with the calcium-binding ATPase date of Potter and Gergely (1975) and that the calcium-magnesium sites played only a structural role for the association of troponin-C with troponin (Cox et al., 1981; Zot and Potter, 1982).

The steric blocking model, in which calcium acts as a simple switch for the actin and myosin interaction, appears to be an oversimplification. Evidence obtained during the inception of the steric blocking model indicated that the regulatory proteins, tropomyosin and troponin, were a tightly coupled complex (Bremel and Weber, 1972). During rigor, bonds between myosin and actin were capable of moving tropomyosin out of the blocking position. Potentiation of the actomyosin ATPase, even below activating levels of calcium, was observed to occur when the MgATP concentration was low. Furthermore, the affinity of calcium for troponin increased under these conditions (Bremel and Weber, 1972), showing that the complex was tightly coupled. Calcium binding to troponin allows tropomyosin movement and, likewise, for the reverse reaction (detailed balance of the reactions of activation and relaxation), movement of tropomyosin increases calcium binding to troponin. Thus formation of rigor complexes, such as under conditions where the ATP concentration is low, would prevent relaxation from occurring since tropomyosin would be prevented from returning to the blocking position by these rigor complexes and, as such, the system would remain "turned on" even at low calcium levels. The above studies supported the steric blocking model but indicated that the model was too simplistic in its approach.

Several lines of evidence suggested that tropomyosin may not physically block the interaction of myosin with the thin filament. It appears difficult for the steric blocking model to explain the increase in binding of tropomyosin to actin in the presence of rigor complexes (Eaton, 1976; Rosenfeld and Taylor, 1985b). When troponin is absent, tropomyosin partially inhibits the ATPase as if tropomyosin were neither in a blocking nor activating position (Eaton et al., 1975). Likewise, the increases in V_{MAX} of the actin•myosin ATPase (Bremel et al., 1972) or the increase in binding of myosin S-1 or myosin•ADP [S-1 or heavy meromyosin (HMM)] to actin when tropomyosin is present suggests that changes may occur within the thin filament upon contraction, or that a

Figure 5



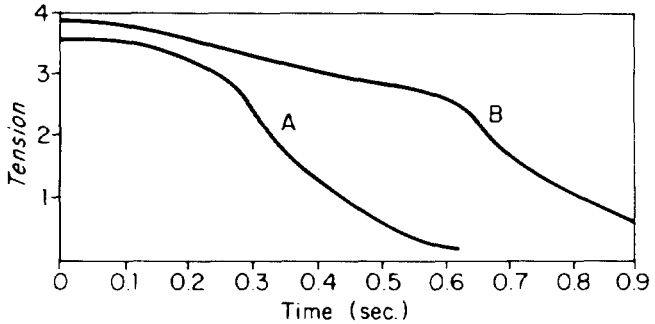
The Hill, Eisenberg and Greene model of muscle contraction regulation. This diagram represents the essential features of their model. Conformational changes of troponin are indicated by shape. Troponin undergoes a conformational change when calcium binds to the regulatory sites, as depicted by the change in shape from a circle to a square, allowing the troponin-tropomyosin complex to change position. Tropomyosin movement affects neighboring tropomyosins through end-to-end interactions which results in a conformational change in troponin, indicated by the square/circle shape. In the absence of calcium, a similar process occurs with cross-bridge binding but the resulting troponin conformational change may not be the same as when calcium binds. Cross-bridge binding in the presence of calcium results in a further movement of tropomyosin. (Adapted from Hill et al., 1983. Proc. Natl. Acad. Sci. USA 80:60)

composite site of actin-tropomyosin is necessary for contraction. The latter concept is supported by evidence indicating two binding states, weak and strong, and thus two binding sites or positions. The rapid equilibria observed for the attached/detached states of myosin (see actin•myosin•ATP interaction) are much easier and quicker to regulate than the equilibrium between the attachment and detachment of states implied by the steric blocking model (Eisenberg and Greene, 1980; Eisenberg and Hill, 1985). Furthermore, the implied irreversibility of attachment/detachment appears not to be an all or none phenomena. This concept is particularly evident when the affinity of troponin for calcium increases at low ATP by the presence of rigor cross-bridges (Bremel and Weber, 1972) which, by the reversibility of the activation sequence, describes a "sometimes all or none" condition.

A model was proposed by Hill et al. (1983) which accounts for the above observations and discrepancies. Figure 5 schematically illustrates the essentials of this model. The number of cross-bridges (one myosin or two heads) per regulated unit is two, as calculated from both structural studies of the filaments and the number of cross-bridges per repeat of the thick filament (Yates and Greaser, 1983). Calcium binding to one regulated unit affects the regulated units on either side of it through the end-to-end interactions of neighboring tropomyosins, as indicated by the movement of tropomyosin in Figure 5, and, in addition, activates the contractile process by enabling the cross-bridges to cycle at a physiological rate. Cross-bridge binding further moves or interacts with tropomyosin in the process, and, in turn, affects each neighboring unit by increasing the binding of both cross-bridges and calcium, as indicated by the change in shape of troponin illustrated in Figure 5. This model accounts for the cooperativity in both myosin (Greene and Eisenberg, 1980; Williams and Greene, 1983) and calcium (Grabarek et al., 1983) binding to the thin filament. Equally important, this model provides a means of describing both the increased troponin affinity for calcium when rigor cross-bridges are present and the activation observed in the absence of calcium at low concentrations of ATP (Bremel and Weber, 1972).

The least understood process in regulation, as noted by both Taylor (1979) and Adelstein and Eisenberg (1980), is the process of relaxation. They reasoned that relaxation may not occur or occurs very slowly under true isometric conditions since bound cross-bridges would keep the thin filament in an activated state. Huxley and Simmons (1972) observed (curve A, Fig. 6) that when a fiber relaxes from an isometric tetanic contraction, two exponential phases were apparent, one slow and the other much faster. In addition, sarcomere lengthening of one end of the fiber was associated with the

Figure 6



Force decline during relaxation. A, Muscle fiber held stationary during relaxation from a tetanus. B, The middle portion of the same fiber is kept at constant length by a servo system and spot-follower. Two phases are present in both curves; a slow and fast exponential phase. The shoulders for both curves were associated with rapid sarcomere lengthening at the fiber ends. (Redrawn from Huxley and Simmons, 1972. Cold Spring Harbor Symp. Quant. Biol. 37:669)

faster relaxation phase so at this point the fiber was no longer isometric. When instrumental measures were taken to carefully control sarcomere length, the slow phase was prolonged (curve B, Fig 6) until the apparatus could no longer control sarcomere length. This implies that relaxation under truly isometric conditions probably occurs very slowly because of the large proportion of force-producing cross-bridges formed and that faster relaxation occurs with movement when the number of cross-bridges is reduced.

Physiological Studies on Calcium Activation

The study of the regulation of actin•myosin•ATP interactions in force or shortening has involved a hierarchical approach from whole-muscle preparations down to such simplified preparations as isolated proteins. Whole muscles afford rather easy dissection and attachment to force and displacement transducers. Yet, mechanical properties are best studied with single fibers obtained by carefully dissecting all but one of the fibers away from a muscle. Contraction is then elicited either by stimulating the attached nerve or passing current from external electrodes across the sarcolemma to obtain either a twitch, using a single stimulus, or tetanus, using a train of stimuli. In addition, the muscle or fiber may be activated by adding drugs to the bathing solution, such as caffeine, or changing the ionic composition of the bathing solution so that the membrane becomes depolarized.

Single-muscle fibers provide a means of obtaining a more simplified preparation when the sarcolemma is either mechanically removed or made permeable by chemical means, leaving the myofilament-protein lattice relatively intact. The earliest preparation of this sort was glycerinated psoas muscle (Szent-Gyorgyi, 1949) prepared by extracting the sarcoplasmic proteins and other soluble constituents with a 50% glycerin and 50% rigor salt solution. Other chemical methods involve treating the fiber or small-muscle bundle with detergents to extract phospholipids (Julian, 1971) or making the membrane semipermeable with chelating agents (Wood et al., 1975). Moreover, the sarcolemma may be mechanically removed by carefully "snagging" it with fine forceps and peeling the membrane back (Natori, 1954). These preparations allow the contractile component to be exposed and equilibrate with the solution the fiber is bathed in. The bathing solutions do differ from the "mother liqueur" which originally bathed the myofibrillar matrix in that many of the physical characteristics such as osmolarity, ionic strength and Donnan distributions are different. However, the chemical environment and thus the experimental protocol may be controlled so that, for example, when skinned fibers are bathed in a solution containing ATP and sufficient calcium, the fibers contract. The intermediate nature of the skinned fiber not only allows the ionic constituents to be studied individually, but proteins may be selectively extracted and even reinserted so that the physiological role of this protein may be studied.

The next step down in the hierarchical approach to studying muscle activation uses isolated proteins. The components of interest are extracted from muscle. This is more of a biochemical approach but physiological studies of contraction involved ingenious use of preparations such as actomyosin threads to measure force (Crooks and Cooke, 1977) and other properties which are more amenable to these preparations. Much of our basic understanding of the calcium activation of the contractile component has come from studies of the isolated proteins.

Studies using skinned fibers provided significant insight into the nature of calcium activation of muscle contraction. The primary reason for this success is the ability to change the level of free calcium which bathes the myofibrillar lattice of the skinned fiber by simply changing the solution which bathes the fiber. In addition, these preparations are amenable to a variety of experimental devices, for example, to monitor force or shortening velocity, structural changes by x-ray diffraction, calorimetric changes associated with nucleotide metabolism or even conformational changes of labeled proteins by fluorescence spectroscopy. Consequently, many of the characteristics of contraction may be studied, in particular, calcium activation by simply controlling the free calcium concentration and hence the level of activation.

Bathing Solutions

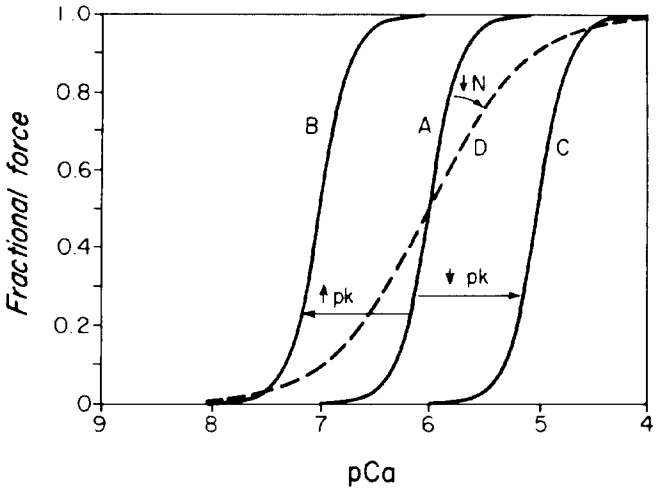
The solutions used in skinned fiber studies usually consist of a potassium salt, pH buffer, ATP including an ATP-regenerating system, magnesium and calcium. The level of calcium is controlled by EGTA buffer system, or other metal chelator, while the level of magnesium is usually buffered by ATP. The composition of the solutions is determined by computer programs which take into account the multiple equilibria between magnesium, calcium and hydrogen ions with the various forms of EGTA, ATP and pH buffer. Equally important, the binding constants are adjusted to reflect the desired ionic strength and temperature. Ionic strength can be kept constant with the ionic form of the pH buffer so that other solution constituents may be changed without affecting ionic strength, a factor which has confounded many results. These solutions do differ with respect to the intracellular fluid which once bathed the myofibrillar matrix of the fiber and as such, the physiological significance of an observation may hinge on the solutions used. Moreover, laboratory to laboratory variation in solution composition and the constants used in computer programs make quantitative comparisons difficult. The benefits, however, of the information obtained from skinned fibers far outweigh the disadvantages and much has been gained and will be gained in the future with this preparation.

Force-pCa Relationship

A majority of the studies on calcium activation with skinned fibers have measured force as a function of calcium (usually expressed as free calcium or pCa, the negative log of the free calcium concentration) while other solution variables are changed. The force-calcium relationship (see Fig. 7) is usually fitted with a relationship described by Hill (1910) to describe the sensitivity and responsiveness of the preparation. Though this formalism may not be appropriate for the analysis of calcium activation, this mathematical description does, however, provide a basis for describing and comparing force-pCa curves.

$$Y = \frac{(KC)^N}{1 + (KC)^N}$$

Figure 7

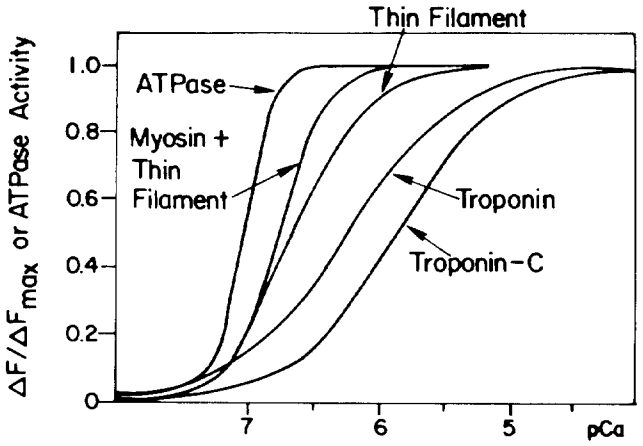


Theoretical curves of the relative isometric force-pCa relationship showing the effect of $pCa_{1/2}$ (pK) and N. The cumulative binding constant, $pCa_{1/2}$, for curves A, B and C are 6.0, 7.0 and 5.0, respectively, with N = 3. The Hill coefficients, N, for curves A and D are 3.0 and 1.0 with $pCa_{1/2} = 6$. Increases in calcium sensitivity are indicated by the increase in pK while a decrease in N, a decrease in cooperativity. Curves were generated from the Hill formalism (1910, J. Physiol. (Lond.) 40:iv) $Y = (KC)^N/[1 + (KC^N]$.

Fitting a force-pCa relationship that is expressed in terms of the fraction of maximal force, Y, and the free calcium concentration, C, with this equation yields two parameters; the Hill coefficient, N, and a cumulative binding constant, K. The cumulative binding constant is defined as the concentration of calcium at the midpoint of the response, $Y = ½$. Since calcium is usually expressed as pCa, the parameter K is expressed in a similar fashion, $pCa_{1/2}$, and is typically in the range of 6.2 to 5.4. But since binding per se is not being measured, this parameter has provided a convenient means of describing the sensitivity of the preparation. As depicted in Figure 7, a decrease in sensitivity would shift the force-pCa curve to the right (curve A → curve B, Fig. 7) with a concomitant decrease in the $pCa_{1/2}$. An increase in sensitivity would produce the opposite effect, a shift of the curve to the left (curve A → curve C, Fig. 7) and an increase in $pCa_{1/2}$. The Hill coefficient N, slope at $pCa_{1/2}$, is generally taken as a measure of cooperativity and typically ranges from 2.0 to 6.0. This parameter was interpreted as a measure of the number or the minimum number of binding sites but the meaning of this constant cannot be formulated into terms of direct binding since the fraction of calcium sites occupied is not directly measured and the response may not be proportional for each bound calcium. Yet, this parameter can be taken as a useful measure of the responsiveness. A Hill coefficient of one has been implied to mean that the binding of one calcium has little or no effect on subsequent binding of calcium. A value less than one may be interpreted as subsequent calciums having a reduced propensity to bind and a value greater than one, a much higher propensity to bind. In terms of responsiveness, a decrease in N or the Hill coefficient (curve A → curve D, Fig. 7) indicates that the fiber requires a greater increase in the concentration of calcium to elicit the same force increment. Thus the fiber described by Curve A, N = 3.0, requires approximately 0.8 pCa units to go from 0 to 50% maximum force while the fiber described by curve D, N = 1.0, requires 2 pCa units to elicit the same relative force response. These parameters do not describe the amount of force but the relative force produced for a given calcium concentration. Thus an additional parameter is required, the maximal calcium-activated force. The Hill formalism used to describe the force-pCa curves of skinned fibers does provide a useful handle for comparison but caution must be exercised in the interpretation of parameters of this type.

The use of this formalism is particularly evident in the work of Grabarek et al. (1983). Figure 8 shows a plot of the fluorescent change which occurs upon calcium binding to fluorescently labeled troponin-C. When calcium binds to isolated troponin-C or troponin, there is no cooperativity in calcium binding i.e., binding of the calcium does not influence the binding of the subsequent calcium. However, the slope at the midpoint increases when the fluorescently labeled troponin-C is incorporated into regulated thin filaments, especially when myosin is present. The increased slope or cooperativity upon calcium binding show that the bound calcium at the calcium-specific sites increases the affinity of calcium binding for subsequent calciums i.e., regulated thin filaments are more responsive to calcium. From this and other carefully controlled experiments, Grabarek et al. (1983) concluded that calcium binding to one regulated unit increased the affinity of neighboring troponins for calcium through the end-to-end interaction of neighboring tropomyosins as depicted in Figure 5.

Figure 8



Effect of the thin filament proteins on calcium titration of the calcium-specific sites of troponin-C as monitored by fluorescently labeled troponin-C. The dansylaziridine label on troponin-C acts as a fluorescence reporter for calcium binding to the calcium-specific sites. The fluorescence was monitored during the calcium titration of the indicated protein or complex. For comparative purposes, the fractional ATPase activity is shown. (Redrawn from Grabarek et al., 1983. J. Biol. Chem. 258:14098)

Similar conclusions on the cooperative behavior of calcium binding to troponin were obtained by Greaser and Moss (1984), and Brandt et al. (1984). Removal of magnesium or calcium at low ionic strength results in the selective extraction of troponin-C from the thin filament (Cox et al., 1981; Moss et al., 1982; Zot and Potter, 1982; Yates et al., 1985). Partial removal of troponin-C from a skinned fiber results in a decreased force. The force is most easily recovered by simply bathing the fiber in a relaxing solution containing troponin-C. The cooperative nature of force development (the Hill coefficient, N) decreased with troponin-C removal and returned to its preliminary value on troponin-C reincorporation. These results suggest that removal of troponin-C decreases the cooperativity by removing neighboring troponin-Cs because the probability of interaction between the regulatory units is decreased (Brandt et al., 1984; Greaser and Moss, 1984).

Factors Affecting the Sensitivity of the Force-pCa Relationship

In retrospect, there are many factors which affect the manner in which a fiber would respond to calcium. From the kinetic studies, an increase in the actin•myosin interaction would most likely affect the amount of force development since, from the length-tension relationship, the number of cross-bridges determine the amount of force developed. Additionally, any factor affecting the binding of calcium would most likely shift the sensitivity of the force-pCa relationship. These predictions are realistic because both regulation and contraction are very tightly coupled and, as such, provide many interesting interactions.

Ionic strength

Increasing the ionic strength from very low concentrations to very high concentrations causes both the calcium sensitivity and maximum calcium-activated force to decrease in practically all muscle fiber types for both vertebrates (Gordon et al., 1973; Thames et al., 1974; Julian and Moss, 1981; Brandt et al., 1982; Kentish, 1984; Fink et al., 1985) and invertebrates (April et al., 1968; Ashley and Moisescu, 1977). The shifts in $pCa_{1/2}$ were more pronounced for red than for white fibers (Fink et al., 1985) with cardiac fibers being very similar to red fibers (Kentish, 1984). Conversely, the maximum activated force was depressed more for white than for red and cardiac fibers (Kentish, 1984; Fink et al., 1985). The essential effect of ionic strength on force was even observed with actin•myosin threads (Crooks and Cooke, 1977).

However, these effects were confounded by the fact that ionic strength is usually adjusted by changing the concentration of potassium. This leads to increases in both potassium and ionic strength. Several studies investigated the effects of a specific salt or ionic strength and found that there were anion, cation and ionic strength effects on both the force developed and on the shifts in $pCa_{1/2}$ (Gordon et al., 1973; Ashley and Moisescu, 1977; Kentish, 1984; Fink et al., 1985). Decreasing the ionic strength resulted in enhanced calcium-activated maximum forces (Gordon et al., 1973) with steeper force-pCa curves (increased cooperativity as determined from Hill coefficients, N) (Kentish, 1984; Fink et al., 1985). Decreasing the concentration of neutral salts shifts the force-pCa curve to a region of greater calcium sensitivity (Kentish, 1984; Fink et al., 1985). Because of the divergent properties observed, two mechanisms were proposed.

The decline in calcium-regulated force with increasing ionic strength was attributed to a change in the interaction of myosin and actin. The affinity of myosin S-1 for actin in both the strong and weak binding states increases as the ionic strength decreases even in the presence and absence of nucleotide (Chalovich and Eisenberg, 1982; Greene et al., 1983). As postulated by Eisenberg and Greene (1980), an increase in binding would appear to not only increase the time spent exerting force but would also increase the force per cross-bridge. Physiological measurements of the number of attached cross-bridges indicate that the proportion of weakly bound cross-bridges increased in relaxed skinned fibers as the ionic strength decreased (Brenner et al., 1984; Reiser and Moss, 1985). X-ray diffraction measurements indicate that with decreasing ionic strength a movement of mass from the thick filament to thin filament was observed in relaxed fibers. This movement of mass correlated with the fraction of low ionic strength cross-bridges bound as determined by stiffness (Brenner et al., 1984; Matsuda and Podolsky, 1984). This observation accounts for the increased resting force, a noncalcium-activated force, observed in skinned fibers when the ionic strength is quite low (See Gordon et al., 1973; Loxdale and Tregear, 1983). Furthermore, Reiser and Moss (1985) recently found that as the ionic strength decreased, the force per cross-bridge increased in the activated skinned fiber. However, maximum shortening velocity shows no dependence on ionic strength (Julian and Moss, 1981). Though difficult to perceive in light of the above, the cross-bridge model of Eisenberg and Greene (1980) predicts this behavior since maximum shortening velocity would depend only on the rate of product release (Siemankowski et al., 1985; Stein et al., 1985). The dependence of maximum force on ionic strength appears to arise from a change in the rate constants of the cross-bridge cycle.

The change in $pCa_{1/2}$ associated with neutral salts was attributed to the competition between calcium and the ionic forms of the salt for the calcium-specific sites (Ashley and Moisescu, 1977; Fink et al., 1985). Several studies, both calcium binding (Fuchs, 1974; Rosenfeld and Taylor, 1985b) and thermodynamic (Tsalkova and Privalov, 1985), show no effect of monovalent ions or ionic strength on calcium binding to the calcium-specific sites of troponin-C, troponin, or regulated thin filaments. Other lines of evidence, however, show an effect of potassium and ionic strength on the interaction of troponin-C with the other troponin subunits and even tropomyosin-troponin with the thin filament (Hartshorne and Mueller, 1968; Eaton et al., 1975; Zot and Potter, 1982; Loxdale and Tregear, 1983; Rosenfeld and Taylor, 1985a). These studies suggest that the degree of coupling of the conformational change in troponin-C or the binding of tropomyosin•troponin to the thin filament for the initiation of contraction shifts the force-pCa curve. A strong coupling would allow for better control through tropomyosin while a weak coupling would, in effect, loosen the regulatory mechanism and allow contraction to occur at a lower concentration of calcium.

Sarcomere length

There is increasing evidence which suggests that changing the sarcomere length modifies both the amount of excitation calcium released from the sarcoplasmic reticulum and the calcium sensitivity of the force response of skinned fibers. There is ample evidence for the latter, most of which comes from skinned fiber studies, but the evidence for the former is far less convincing and as such, emphasis will be placed on the calcium sensitivity changes associated with sarcomere length changes. Additional information on this topic can be found in the review by Stephenson and Wendt (1984).

Increasing the sarcomere length beyond maximum filament overlap was found to increase the apparent calcium sensitivity in practically all muscle fiber types (Endo, 1972, 1973; Fabiato and Fabiato, 1978b; Moisescu and Thieleczek, 1979; Hibberd and Jewell, 1982; Stephenson and Williams, 1982, 1983; Moss et al., 1983) with the exception of amphibian slow fibers (Stephenson and Williams, 1983). As expected from the force-length relationship, maximum calcium-activated force decreases in skinned fibers on either side of the plateau as it does with intact fibers (Moss, 1979; Julian and Moss, 1980). Nevertheless, decreasing the sarcomere length from rest length results in an apparent decrease in the calcium sensitivity of force (Hibberd and Jewell, 1982). The quantitative characteristics of the length-dependent sensitivity changes depend on fiber type with more of a shift occurring with both red and cardiac fibers than with white or mixed fibers (Fabiato and Fabiato, 1978b; Moisescu and Thieleczek, 1979; Stephenson and Williams, 1982).

Several molecular mechanisms have been proposed to explain the $pCa_{1/2}$ dependency on length, yet there was not much evidence to substantiate them (for further discussion of these mechanisms, see Stephenson and Wendt, 1984). One proposed mechanism postulates that since the interfilament spacing decreases with increasing sarcomere length, cross-bridges would be closer to the thin filament. As such, potentiation of thin filament activation would occur by altering the attachment-detachment rates of the cross-bridge (Endo, 1973; Maughan and Godt, 1981). The observed shifts in $pCa_{1/2}$ could not be accounted for, however, when the lattice was compressed osmotically with high molecular weight polymers to bring the filaments closer (Fabiato and Fabiato, 1978b; see also Maughan and Godt, 1981). Even when the concentration of MgATP was decreased, to slow down cross-bridge kinetics, the effect was not modified (Stephenson and Williams, 1983).

Careful analysis by Moss et al. (1983) of the Hill plots of force-pCa curves exhibiting length-dependent $pCa_{1/2}$ shifts demonstrated that at longer sarcomere lengths the curve was monophasic while at shorter sarcomere lengths (2.4-2.6 μm), a biphasic curve was obtained. Interpretation of these results suggested that less calcium is required for the stretched sarcomere lengths than at shorter sarcomere lengths. They postulated that an additional lower affinity site was required for force development at the shorter sarcomere lengths which possibly involved myosin or its phosphorylation.

Temperature

Lowering the temperature results in an increase in apparent calcium sensitivity of force. This phenomenon was observed for both red (Stephenson and Williams, 1985) and white fibers (Stephenson and Williams, 1981; Godt and Lindley, 1982). In contrast, crustacean fibers did not exhibit a change in sensitivity (Ashley and Moisescu, 1977), while crayfish fibers showed a sensitivity decrease with decreasing temperature (Orentlicher et al., 1977). In all cases, however, the maximal calcium-activated force developed by the fibers diminished with decreasing temperatures. Mammalian fibers had minute, if any, force development at 0° to 1° C (Stephenson and Williams, 1981, 1985). But increases in temperature to 35° C from room temperature did not result in an increased force (Stephenson and Williams, 1981). The observed increase in calcium sensitivity was much greater for white fibers than for red with the latter showing considerable variability. As would be expected, the rates of tension development and relaxation decreased with temperature.

As noted above, the decreased force with temperature could account for many of the observations of the temperature dependence of tetanic force for intact fibers with the dependence becoming very steep below a temperature of 10° C. Many of these observations could not be associated with membrane activation (for further discussion, see Godt and Lindley, 1982). As a result, it appears that the contractile properties of the fiber change rather than temperature-dependent changes occurring with EC-coupling or activation through membrane depolarization. In addition to the lowered force levels with temperature, the increased calcium sensitivity could account for an increase in the resting tension of cat soleus muscle fibers when cooled to 4° C (Hill, 1972). The low Hill coefficient, N, or responsiveness of the force-pCa curve for red fibers coupled with an increased sensitivity at the lower temperature could allow low force development since the curve could be shifted to a region where the resting free calcium would be high enough for activation (Stephenson and Williams, 1985).

The observed decrease in force with lower temperature could be explained by either a decrease in the number of cross-bridges formed, a lower force per cross-bridge or a combination of both. Biochemical studies show that the association constant of myosin for actin is highly temperature dependent with the affinity being reduced at the lower temperatures (Highsmith, 1977) which suggests a decrease in the force per cross-bridge. Physiological studies indicate that the number of cross-bridges, as measured by stiffness, does not change with temperature in maximally activated-skinned fibers (Kuhn et al., 1979). Studies on intact fibers indicated that the force per cross-bridge decreases with temperature (Ford et al., 1977). Furthermore, intriguing kinetic studies with skinned fibers showed that the attachment rate is more temperature dependent than detachment (Goldman et al., 1984a). In addition, the reduced force at lower temperatures accompanied by continued hydrolysis of ATP indicates that a temperature-dependent change within the cross-bridge reduces the coupling efficiency of hydrolysis with the work stroke (Stein et al., 1982). Several lines of evidence suggest that a conformational change may occur in the nucleotide•cross-bridge when the temperature is reduced (for further discussion, see the review by Shriver, 1984). Together, these results indicate that the reduced isometric tension at lower temperatures occurs from the force per cross-bridge

being decreased rather than a reduction of the number of cross-bridges.

Godt and Lindley (1982) attributed the increased calcium sensitivity at lower temperatures to the temperature dependence of calcium binding to troponin-C. The affinity of calcium for troponin-C increases as the temperature declines (Potter et al., 1977) and as such, the calcium sensitivity would be predicted to increase as the temperature decreased. The calculated free energy change ($\triangle G$) and enthalpy ($\triangle H$) obtained from the force-pCa curves at different temperatures were similar to the values obtained with calcium binding to the calcium-specific sites of troponin-C (Godt and Lindley, 1982). Further support for their conclusion was obtained when the magnesium was lowered in the activating solutions, shifting the calcium sensitivity (see below) which also resulted in comparable temperature-dependent $pCa_{1/2}$ shifts. Since calcium competes for magnesium at the calcium-magnesium sites, they concluded that the temperature dependence of the force-pCa relationship was associated with calcium binding to the calcium-specific sites. The agreement of the thermodynamic variables may be fortuitous in that troponin-C is only one protein in the regulatory complex. This complex exists as a tightly coupled unit and as such, temperature-dependent changes could also occur elsewhere. Further research is needed on the temperature dependence of calcium binding to troponin-C of other fiber types to ascertain whether the predicted shifts correlate with the shifts in calcium sensitivity.

MgATP

Nucleotides and the products from its hydrolysis have been used in an attempt to link the kinetics of the actin•myosin•ATP interaction with mechanical studies on force and velocity of shortening. These studies have been performed both in the presence and absence of calcium in order to gain information on the role of the myosin conformational states in muscle contraction. The effect of nucleotides and byproducts on the force-pCa curve were also investigated to ascertain the role of the cross-bridge in modulating calcium activation.

Decreasing the MgATP from physiological levels (1-5 mM) to 50-100 μM was found to increase the calcium sensitivity of force development of many muscle types studied: skeletal muscle fibers (Godt, 1974; Fabiato and Fabiato, 1975; Orentlicher et al., 1977); cardiac muscle (Fabiato and Fabiato, 1975, 1978b; Best et al., 1977) and crustacean muscle fibers (Reuben et al., 1971; Brandt et al., 1972; Kawai and Brandt, 1977). The results are consistent with the Bremel and Weber (1972) hypothesis in that lowering the MgATP concentration would lead to an increase in a proportion of rigor cross-bridges which, through cooperativity effects, would result in an increase in the affinity of troponin for calcium as depicted in Figure 5. This would decrease the level of calcium required to activate contraction and as such, would shift the force-pCa curve toward increased sensitivity.

In the absence of calcium, decreasing the MgATP concentration to lower levels in relaxed fibers results in an increase of resting or noncalcium-regulated force to a magnitude which becomes greater than the maximum calcium-activated force obtained at physiological levels of MgATP. Reducing the MgATP levels further results in a decline in tension to rigor levels. This observation was observed for white (Godt, 1974; Loxdale and Tregear, 1983; Ferenczi et al., 1984a; Goldman et al., 1984a; Moss and Haworth, 1984), red (Krasner and Kushmerick, 1983; Loxdale and Tregear, 1983), cardiac (Fabiato and Fabiato, 1975; Best et al., 1977), and crustacean muscle fibers (Reuben et al., 1971; Brandt et al., 1972; Kawai and Brandt, 1977). The concentration of MgATP at which peak tension occurs in the absence of calcium appears muscle specific, with white muscles requiring much less MgATP than red or cardiac. The velocity of shortening in the absence of calcium activation paralleled the MgATP dependency of force. In raising the levels of MgATP from rigor levels, the velocity increased and then decreased (Moss and Haworth, 1984). It appears that the increase in the proportion of rigor bonds activates the contractile component even in the absence of calcium. Careful kinetic analysis revealed that cross-bridge detachment becomes rate limiting at low levels of MgATP (Ferenczi et al., 1984a; Goldman et al., 1984a; Moss and Haworth, 1984). The addition of calcium results only in a slight decrease of the force as MgATP levels approach physiological levels, indicating that the system was activated even in the absence of calcium (Ferenczi et al., 1984a; Goldman et al., 1984b). This implies that cross-bridge detachment is not influenced by calcium since both force and shortening occurred as an active process in the absence of calcium. Thus, the regulatory process facilitates cross-bridge binding though product release, but the process does not influence cross-bridge detachment (Ferenczi et al., 1984b). This lends further support to the observation of Huxley and Simmons (1972), that relaxation, in a true isometric state, would occur slowly.

pH

Changing the pH of the solution which bathes a skinned fiber has profound effects on the force-pCa relation and the amount of maximum calcium-activated force. The shift in $pCa_{1/2}$ toward a decreased calcium sensitivity, with a drop in pH from 7.0-7.4 to 6.5-6.2, was greater for red (Donaldson and Hermansen, 1978) and cardiac fibers (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978a) than for white fibers (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978; Robertson and Kerrick, 1979). Decreasing the pH even further resulted in a further shift in calcium sensitivity with the force becoming zero between pH 5.0-5.5 (Robertson and Kerrick, 1979). Force depression was greater for white fibers than with red or cardiac (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978a). However, force depression was not observed in crustacean fibers, yet a shift in the $pCa_{1/2}$ paralleled the observations with other fibers' types (Ashley and Moisescu, 1977).

In contrast to the above, no depression in the actin•myosin ATPase was observed in the pH ranges studied (pH6.2-7.0) for either cardiac myofibrils (Blanchard and Solaro, 1984) or myofibrils isolated from white muscle (Blanchard et al., 1984). However, Godt and Baumgarten (1984) observed a decrease in the ATPase of rabbit psoas fibers at pH 5.0. These results must be interpreted cautiously since the distribution of the various forms of ATP (ATP, MgATP, CaATP, MgHATP, CaHATP, etc.) changes as the pH is lowered and as

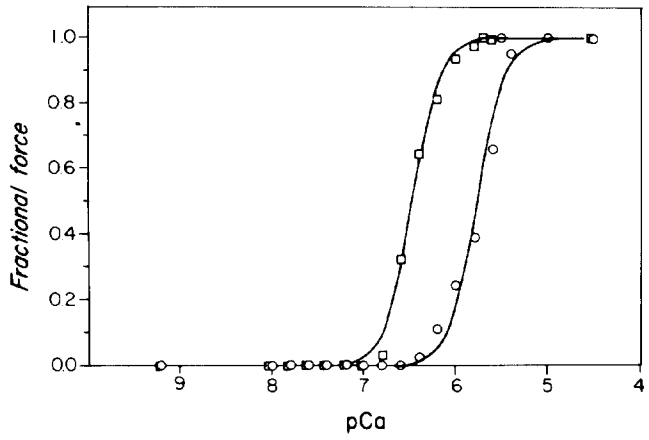
such, one form may be predominantly hydrolyzed over the other as the pH is dropped.

The decrease in calcium sensitivity was attributed to an increased competition of H^+ with calcium for the calcium-specific sites but calcium binding studies in this pH range yielded conflicting results (see Blanchard et al., 1984). This conflict centered on the use of EGTA as a metal buffer in the pH ranges used. The effect was not observed by Fuchs (1974) nor Stull and Buss (1978) when EGTA was left out. However, calcium binding to both cardiac and skeletal myofibrils in the presence and absence of EGTA demonstrated that a decrease in the affinity of troponin for calcium decreased as the pH was lowered (Blanchard and Solaro, 1984; Blanchard et al., 1984). Furthermore, observations made with fluorescently labeled troponin-C, both cardiac and skeletal, exhibited a pH dependence in calcium binding to the calcium-specific sites (Robertson et al., 1978a, 1978b). Even more substantial evidence comes from the crystallographic studies of skeletal troponin-C which show the absence of calcium from the calcium-specific sites but not from the calcium-magnesium sites at pH 5.0 (Herzberg and James, 1985; Sundaralingam et al., 1985).

Magnesium

Decreasing the level of free magnesium in the solutions which bathe the fiber results in an increase in calcium sensitivity as shown in Figure 9. The $pCa_{1/2}$ dependency on magnesium has been observed in skinned white (Donaldson and Kerrick, 1975; Fabiato and Fabiato, 1975; Donaldson and Hermansen, 1978; Godt and Lindley, 1982), red (Donaldson and Hermansen, 1978), cardiac (Fabiato and Fabiato, 1975; Best et al., 1977; Donaldson et al., 1978; Donaldson and Hermansen, 1978), and crustacean fibers (Ashley and Moisescu, 1977). The shift is greater for cardiac or red fibers than for white fibers (Donaldson and Hermansen, 1978). Maximum calcium-activated force was not observed to be dependent on the level of magnesium with the exception of the study by Donaldson et al. (1978). There is no apparent reason for this discrepancy.

Figure 9



Comparison of the relative isometric force-pCa relations at 3 mM and 50 μM free magnesium from Rhod-TnC incorporated fibers. The isometric force is expressed as a fraction of the maximum force for fiber Rh105225 in 3 mM free magnesium (squares), and fiber Rh105235 in 50 μM free magnesium (circles). Approximately 25% of the endogenous troponin-C for each fiber was replaced by rhodamine labeled troponin-C. Curves were fit with the equation $Y=(KC)^N/[1+(KC)^N]$ by nonlinear least squares regression. Solutions at a pH of 7.0 contained 130 mM free potassium, 3 mM MgATP, 15 mM total EGTA with the ionic strength adjusted to 0.195 M with MOPS buffer. Temperature was 10° C.

There are three probable sites of interaction where magnesium could modulate calcium sensitivity: troponin-C, tropomyosin, and myosin. Myosin light chain-2 was shown to bind magnesium as well as calcium. The binding of magnesium or calcium to the myosin light chain-2 and to the LMM-HMM hinge region was studied in relation to cross-bridge activation. There appears to be no physiological role of magnesium or calcium binding to myosin light chain-2 (Robertson et al., 1981). Binding to the LMM-HMM hinge does affect the radial disposition of the cross-bridges by bringing them closer to the backbone of the thick filament. However, significant amounts of magnesium would be required at physiological ionic strength since both calcium and magnesium binding decreases with ionic strength (See Ueno et al., 1983). Furthermore, no changes in force were observed for large changes in magnesium, implying little or no dependence of cross-bridge interaction on magnesium.

Troponin-C has binding sites for both calcium and magnesium, and, in particular, a class of sites which bind both magnesium and calcium competitively. As such, this provides an attractive hypothesis to explain the calcium sensitivity dependency on magnesium. However, the calcium-specific sites were implicated as the regulatory sites and most likely, any magnesium effect would be precluded since these sites bind only calcium (Potter et al., 1981). There are several studies which suggest interaction between the two classes of sites (Wang et al., 1983; Ellis et al., 1984; Tsalkova and Privalov, 1985; Rosenfeld and Taylor, 1985a) which could conceivably increase the apparent affinity of the regulatory sites for calcium. In contrast, structural changes occurring in troponin-I when calcium binds to the calcium-specific sites of troponin-C are independent of the metal at the calcium-magnesium sites (Leavis et al., 1984; Strasburg et al., 1985). The extent of interaction, if any, especially when troponin-C is incorporated within a thin filament, is unknown.

The binding of tropomyosin to F-actin increases as the level of magnesium and ionic strength increases with magnesium having a more profound effect than ionic strength (Eaton et al., 1975; see also Lehrer and Morris, 1982; Loxdale and Tregear, 1983). Reducing magnesium concentration diminishes the ability of tropomyosin to inhibit the actin•myosin ATPase (Eaton et al., 1975) suggesting that tropomyosin is bound weakly. It also suggests that less energy is required to move tropomyosin from an inhibition to an activation state. These observations are further supported by the inhibition of a high resting force at low MgATP as magnesium is increased (Reuben et al., 1971; Best et al., 1977; Loxdale and Tregear, 1983). Moreover, when binding of tropomyosin to F-actin is weak (conditions of low magnesium and ionic strength) the addition of rigor cross-bridges strengthens the binding of tropomyosin to actin (Eaton, 1976). This would appear to be a plausible explanation for the observed shift of force with calcium at low magnesium.

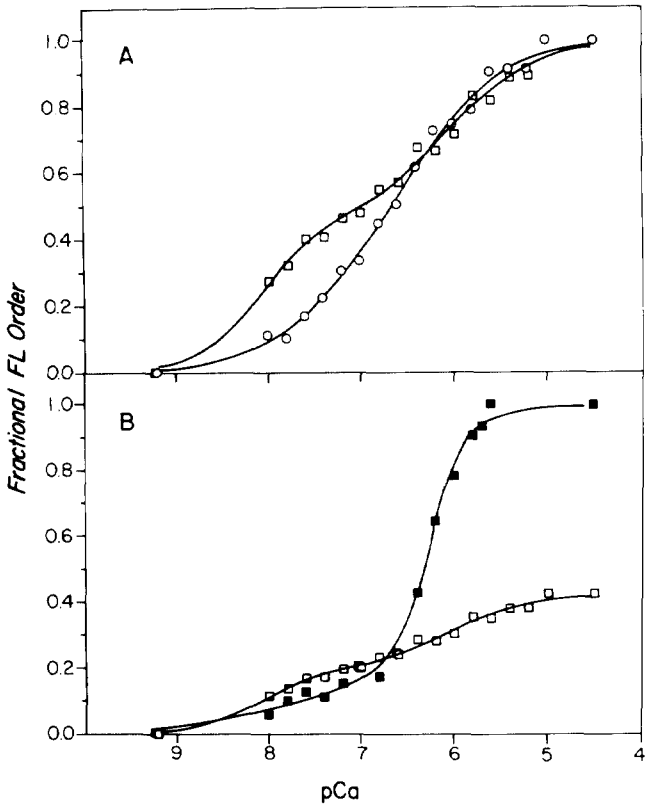
We investigated thin filament activation at low and physiological levels of magnesium using a fluorescent probe, rhodamine, attached to troponin-C (Yates et al., 1985). Development of two important techniques provides handles for the understanding of the sequence of events involved in the regulation of contraction. One, the selective extraction of troponin-C and its reinsertion into skinned-skeletal fibers (Moss et al., 1982; 1983). Two, the ability to detect calcium binding to troponin-C. Metal binding to these sites was studied by using a fluorescent method sensitive to the conformational changes which occur on binding. Several different fluorescent probes were used which detect binding to the calcium-specific sites (Johnson et al., 1978; Grabarek et al., 1983) and binding to the calcium-magnesium sites (Grabarek et al., 1983; Rosenfeld and Taylor, 1985a, 1985b; Yates et al., 1985). Studies involving the use of these probes have provided a wealth of information on the sequence of events and the kinetics of calcium activation of muscle contraction. Consequently, troponin-C could be removed from a skinned fiber, selectively labeled, and reintroduced into the fiber to monitor the conformational changes which occur with the labeled protein.

Incorporation of the labeled troponin-C (Rhod-TnC) into skinned fibers does not affect the force-pCa relation nor the maximum calcium-activated tension (Yates et al., 1985). Decreasing the free magnesium results in an increased calcium sensitivity as discussed previously. Preliminary studies using incorporated Rhod-TnC into fibers beyond overlap, i.e., regulated thin filaments, show a biphasic fluorescent change when the fiber is titrated with calcium at low magnesium (50 μM) (Fig. 10a). The $pCa_{1/2}$, as obtained from nonlinear-least squares regression, are compatible with the binding constants of both the calcium-specific sites ($pCa_{1/2} = 6.2$) and the calcium-magnesium sites ($pCa_{1/2} = 8.2$) at 50 μM of free magnesium (Potter and Gergely, 1975). Raising the concentration of magnesium to more physiological levels (3 mM) results in a calcium titration curve which shows less biphasic characteristics (Fig. 10a). Because there would be more magnesium to compete for calcium in binding to the calcium-magnesium sites, there was a dramatic shift in the $pCa_{1/2}$ of the calcium-magnesium sites to a much higher calcium level ($pCa_{1/2} = 7.4$). But there appears to be no significant shift in the $pCa_{1/2}$ associated with the calcium-specific sites ($pCa_{1/2} = 6.1$), contrary to the hypothesis of a magnesium affect on calcium binding to the calcium-specific sites.

Similar observations are seen when rigor cross-bridges are present with full overlap of thick and thin filaments (results not shown). The major magnesium-dependent shift in $pCa_{1/2}$ occurs with the calcium-magnesium sites. However, there is a slight increase in cooperativity and in calcium sensitivity associated with the calcium-specific sites consistent with the Bremel and Weber (1972) hypothesis and the observation of Grabarek et al. (1983) that cross-bridge interaction increases the calcium-binding affinity of troponin. Thus when magnesium was decreased, the only exhibited change was the apparent increased affinity of calcium for the calcium-magnesium sites. Evidently, calcium binding to the regulatory sites cannot account for the observed magnesium-dependent shift of the force-pCa relation.

Comparing the titration curve at low magnesium for the regulated thin filament to the fluorescent changes when force is allowed to develop shows no significant change associated with the calcium-magnesium sites and only a slight shift of the $pCa_{1/2}$ presumed to be the calcium-specific sites (Fig. 10b). However, there is an increase in the magnitude of the fluorescent change in the region associated with calcium binding to the calcium-specific sites (compare Fig. 9 with Fig. 10b). The increased magnitude results from cross-bridge interaction with the magnitude being intermediate between relaxation and that induced by rigor cross-bridges (Yates et al., 1985). Interestingly, there was a dramatic shift of the fluorescent change associated with calcium activation between low and physiological levels of magnesium. Both fluorescent responses closely correlated with force. In brief, there was a magnesium-dependent shift observed with the

Figure 10



Comparison of the fractional fluorescent order from Rhod-TnC incorporated fibers (A) beyond overlap in rigor solutions at 3 mM and 50 μM free magnesium and (C) in overlap with rigor and ATP containing solutions with 50μM free magnesium. The fluorescent order, as determined by linear dichroism, is expressed as the fraction of the maximum change observed. A, Fibers Rh204295 (open circles) and Rh104305 (open squares) were bathed in solutions containing 3 mM and 50 μM free magnesium, respectively. B, Fibers Rh105235 (closed squares, force-pCa record in Fig. 9) bathed in solutions containing MgATP and Rh104305 (open squares) bathed in rigor solutions. The fluorescent order of fiber Rh104305 is expressed as a fraction of the maximum change observed for fiber Rh 105235. Fibers in B were bathed in solutions containing 50 μM free magnesium. Curves were fit with the equation $Y = (KC)^N/[1 + (KC)^N]$ by nonlinear least squares regression. Solutions are the same as those described in Figure 9 with rigor solutions having no added MgATP. Temperature was 10° C.

$pCa_{1/2}$ of the calcium-specific sites during force development. In contrast, no shift in $pCa_{1/2}$ was observed when thin filaments or thin filaments with bound rigor cross-bridges were titrated with calcium at both physiological and low levels of magnesium. This implies that an intervening step or process is affected by magnesium.

The only change observed for the fluorescent changes associated with activation occurred during active force development. Since there was no difference in the magnitude of maximum calcium-activated force nor a change in the Hill coefficient, it would seem that cross-bridges were not affected by the changes in magnesium concentrations. Similarly, the mechanism attributed to the interaction between calcium-magnesium sites and calcium-specific sites seems unlikely in that both the regulated thin filament and the thin filament with bound cross-bridges exhibited little or no change in the $pCa_{1/2}$ of the fluorescence. The $pCa_{1/2}$ obtained during active force development displayed a dramatic magnesium-dependent shift. It seems likely that magnesium-dependent binding of the tropomyosin-troponin complex to the thin filament could account for the observed increase in the force-pCa relations as free magnesium was decreased. Experiments to study this possibility are in progress.

Summary of Skinned Fiber Studies

As reviewed, there are many factors, ionic strength, temperature, length, pH, magnesium and MgATP, which affect the physiological response of a skinned muscle fiber to calcium. Not only is the calcium sensitivity of the force response sensitive to these factors but so is the maximum calcium-activated force. Categorizing the responses to these factors reveals that increasing muscle length from rest length is associated with increases in calcium sensitivity of force. Moreover, decreasing either temperature, ionic strength, MgATP, pH or magnesium from their physiological levels also increases calcium sensitivity. Many of these factors also affect maximal calcium-activated force with increases in length or decreases in either the temperature, pH and ionic strength decreasing the force a fiber develops. Of the aforementioned factors, some also influence cooperativity. Although much suggestive evidence implies that cooperativity is affected, the reported variation precludes the formation of any firm conclusions.

Interestingly, the magnitude of the observed shifts appears to be fiber-type specific. Greater shifts in the calcium sensitivity of force development were observed with the fibers from red and cardiac muscles. On the other hand, those factors which alter maximum calcium-activated force affect white fibers more so than either red or cardiac fibers. This is no doubt a reflection of the differences observed in contractile proteins, their interaction as a tightly coupled complex, and the manner in which chemical energy is expressed as a mechanical event.

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