ALTERATIONS IN THE CA^{2+} SENSITIVITY OF TENSION DEVELOPMENT BY SINGLE SKELETAL MUSCLE FIBERS AT STRETCHED LENGTHS

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ABSTRACT The apparent length dependence in the calcium sensitivity of tension development in skeletal muscle has been investigated in the present study. At sarcomere lengths of 2.46–2.62 μm, the Hill plot of tension-pCa data is well fit by not one but two straight lines, suggesting the possible involvement of more than a single class of Ca^{2+}-binding site in tension development. On the other hand, increasing the sarcomere length to 3.00–3.25 μm yielded Hill plots that were described by a single straight line, which indicates that at long lengths tension might be regulated by the binding of Ca^{2+} to a single class of Ca^{2+}-binding sites, presumably the low affinity sites of TnC. This length-dependent transformation of the tension pCa relation occurred at free Mg^{2+} concentrations of both 0.05 and 3.2 mM. Although the mechanism of this effect is uncertain, plausible explanations for the biphasic Hill plot at the shorter lengths include the possible involvement of Ca^{2+} activation of the thick filaments and/or myosin LC_2 phosphorylation in the process of tension development.

INTRODUCTION

Tension development in vertebrate striated muscle results from the interaction of cross-bridges, which extend from the myosin-containing thick filaments, with sites on the actin-containing thin filaments (see Huxley, 1974, for a review). This interaction is inhibited in the relaxed state due to the presence of the regulatory proteins troponin and tropomyosin on the thin filaments. With excitation and the subsequent rise in myoplasmic calcium levels, calcium is bound to troponin resulting in the disinhibition of the thin filaments (Ebashi and Endo, 1968). Thus, calcium is believed to have a switchlike action in the regulation of muscle contraction, with the proportion of actin sites available for interaction with myosin dependent in a characteristic way upon the level of activator calcium in the myoplasm. However, the idea that the binding of calcium to troponin is the sole regulator of actin-myosin interaction in the zone of thick- and thin-filament overlap may be overly simple. For example, Endo (1972, 1973) has shown that isometric tension development in skinned skeletal muscle fibers at low levels of activator calcium actually increases as the length of the fiber is increased above the optimum, a length range in which tension would be expected to decrease, due to decreased amounts of thick and thin filament overlap (Gordon et al., 1966; Julian and Moss, 1980). This apparent length dependence in the calcium sensitivity of tension development has been investigated further here. Measurements of the relationship between tension and pCa (i.e., $-\log [Ca^{2+}]$) in skinned fibers indicate that alterations in this relationship as a result of changes in sarcomere length occurred only at the highest pCa's studied, rather than as a wholesale leftward shift of this curve at long lengths, as had been reported previously in skeletal (Moisescu and Thieleczek, 1979) and rat ventricular (Hibberd and Jewell, 1982) muscles. Restriction of this effect to low calcium concentrations raises the possibility that calcium activation of striated muscle contraction at sarcomere lengths near the optimum may be a two-step process.

METHODS

Strips of muscle were obtained from psoas muscles of adult male New Zealand rabbits (2.0–2.5 kg body weight) and were stored at −22°C in relaxing solution containing 50% (vol/vol) glycerol for a period of 3–14 d before use (Julian et al., 1981). Single fibers were dissected free in relaxing solution and were then cut into segments 2–5 mm in length. One of these segments was transferred to the experimental chamber and was then attached with small connectors (Moss, 1979) to wires extending from a force transducer (model 401; Cambridge Technology, Inc., Cambridge, MA) and servo motor (model 300 s; Cambridge Technology, Inc.). The mean sarcomere length was adjusted to 2.46–2.62 μm, as determined by direct-light microscopy (Moss, 1979), and the temperature was lowered to 15°C. The solutions used to activate the fiber segment were similar to those described previously (Moss, 1979). Relaxing solution contained 100 mM KCl, 2 mM EGTA, 1 mM $MgCl_2$, 4 mM $ATPNa_2$, and 10 mM imidazole; the pH was 7.00. The composition of the activating solution was identical to that of relaxing solution except that the concentration of EGTA was 4.0 mM and various amounts of $CaCl_2$ were added to yield concentrations of free Ca^{2+} in the range $10^{-5.49}$–$10^{-7.00}$ M. All reagents were obtained from the Sigma Chemical Co. (St. Louis, MO). In some instances, the free magnesium concentration was increased to 3.2 mM, which necessitated a decrease in total ATP to

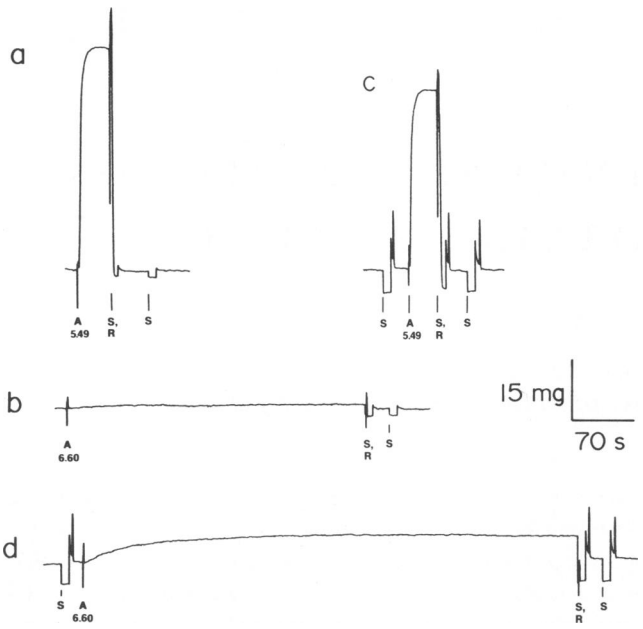

FIGURE 1 Records of tension vs. time following Ca^{2+} activation at optimum and stretched lengths. The following were the mean sarcomere length in micrometers and pCa in each instance: (*a*) 2.51 μm, 5.49; (*b*) 2.51 μm, 6.60; (*c*) 3.01 μm, 5.49; (*d*) 3.01 μm, 6.60. In parts *a* and *b*, the bathing solution was changed from relaxing to activating at the time indicated by *A*. When a steady tension was achieved, the segment was slackened and then relaxed at the time indicated by *S*, *R*. The segment was reextended and resting tension was measured by slackening (*S*) the segment. A similar sequence was used in parts *c* and *d* except that each activation was preceded as well by a resting tension measurement indicated by *S*. Segment no. 42982–2; overall length was 2.05 mm at optimum length; temperature was 15°C. Abbreviations: *S*, slack; *R*, relaxing solution; *A*, activating solution. Each slack step (*S*) was followed by re-extension of the segment to its original length.

maintain a constant concentration of MgATP. The final concentrations of all metals and ligands were determined by solving simultaneously the equilibrium equations for the complexes involved. Apparent stability constants (K_{app}) for the metal-ligand complexes were calculated (Portzehl et al., 1964) from the absolute binding constants listed by Sillen and Martell (1964). The values for K_{app} were as follows: CaEGTA, $10^{6.68}$ M^{-1}; MgEGTA, $10^{1.61}$ M^{-1}; CaATP, $10^{3.60}$ M^{-1}; MgATP, $10^{3.84}$ M^{-1}.

The initial contraction in each fiber segment was done at pCa 5.49 to obtain a measure of the tension-generating capability of the segment, and also to check for sarcomere length uniformity during maximal activation (Moss, 1979). Succeeding activations were done at higher pCa's in the range 5.75–7.00. Every third activation was done at pCa 5.49 to monitor any decline in segment performance. During each contraction, tension was allowed to reach a steady level, upon which the segment was rapidly slackened (step complete within 1 ms) and changed to relaxing solution (Fig. 1). The difference between the steady-state active tension and the tension attained immediately following the slack step was defined as total tension. Resting tension exerted by the segments was measured before or after each contraction using the slack step procedure. Active tension was then calculated as the difference between the total and resting tensions. Tensions (P) exerted during submaximal contractions at long and short lengths were expressed as a fraction of the maximal tension (P_o) developed at a sarcomere length in the range 2.46–2.62 μm in the same segment. At stretched lengths, where resting tension was relatively high, stabilization of resting tension took several minutes due to stress relaxation. It was found that a stable resting tension could be quickly established at these lengths by first stretching the segment a small amount beyond the length of interest and then returning the segment to this length. The experimental records in Fig. 1 *c–d* demonstrate this protocol.

RESULTS AND DISCUSSION

The time courses of tension development at maximal and submaximal calcium levels are shown for one fiber segment in Fig. 1. In this particular instance, at a sarcomere length of 2.51 μm, the steady tension at pCa 5.49 was 1.33 kg/cm^2, normalized to segment cross-sectional area (Moss, 1979). In all segments studied, a maximum steady tension of 1.38 ± 0.26 kg/cm^2 (n = 18) was obtained at sarcomere lengths that varied between 2.46 and 2.62 μm. Lowering the calcium concentration to achieve pCa 6.60 in the activating solution resulted in a decrease in isometric tension to 0.02 P_o (Fig. 1 *b*). Stretching the fiber so that sarcomere length was 3.01 μm resulted in a decrease in the maximum Ca^{2+}-activated tension (Fig. 1 *c*), as would be expected on the basis of decreased thick and thin filament overlap (Gordon et al., 1966); however, steady tension at pCa 6.60 (Fig. 1 *d*) was found to increase substantially relative to that obtained at the same pCa and shorter sarcomere length (Fig. 1 *b*). This phenomenon in rabbit psoas fibers is qualitatively the same as that reported by Endo (1973) in frog skinned muscle fibers.

The relationship between relative isometric tension, expressed as a fraction of P_o, and sarcomere length is plotted in Fig. 2 for four fiber segments. During maximal activation (pCa 5.49), tension decreased approximately linearly as the segment length was increased. This finding is in good agreement with previous results obtained from frog skinned muscle fibers (Julian and Moss, 1980). It should be noted that in contrast to the work involving frog muscle, the fibers of the present study maintained excellent striation uniformity during maximum tension development, thereby precluding the possibility that sarcomere length rearrangements during contraction might distort the form of the length-tension relation at long lengths. Reduction of the free Ca^{2+} to pCa's of 6.60 and 6.70

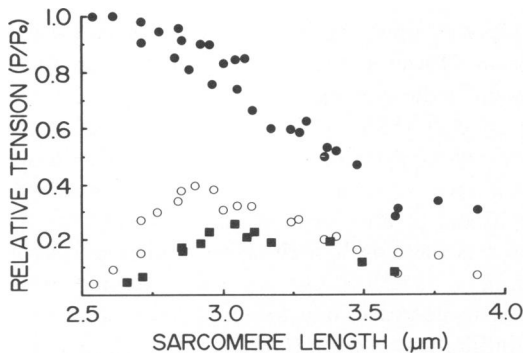

FIGURE 2 Plots of relative tension vs. sarcomere length obtained at pCa of 5.49 (•), 6.60 (○), and 6.70 (■). In each instance the developed tension (P) is expressed as a fraction of the maximum tension (P_o) developed by the same fiber segment at pCa 5.49 and a sarcomere length of ~2.50 μm. Data is from four different fiber segments.

resulted in transformations of the length-tension relation to a form similar to that reported by Endo (1972, 1973) in frog skinned muscle fibers. Tension actually increased as sarcomere length was increased above 2.5 μm, achieving maxima at ~2.8 μm (pCa 6.60) and 3.0 μm (pCa 6.70). Further increases in length in each case resulted in a linear decline in tension, extrapolating to zero at ~4.0 μm.

The effect of sarcomere length upon Ca^{2+}-activated tension was investigated over a wide range of calcium concentrations encompassing the fully relaxed (i.e., pCa 7.00–6.86) and fully activated (pCa 5.49) states. At a sarcomere length of ~2.50 μm, the tension pCa relation (Fig. 3 *a*) appears to be sigmoidal, as has been commonly reported (Hellam and Podolsky, 1969). However, a Hill plot (Hill, 1913) of this same data (Fig. 3 *b*), which is a useful indicator of the form of the tension-pCa relation, is fit by not one but two straight lines that intersect at about pCa 6.50, indicating that at this length a Hill equation with single values for n and k will not describe the data. A Hill coefficient (n), which is the lower limit for the number of sites involved in the binding of Ca^{2+} (Koshland, 1970), was calculated in each case as the slope of the fitted straight line. For pCa < 6.50, n was found to be 1.99, and for pCa 6.50, n was 6.67. A possible explanation for this data is that two classes of binding sites, differing in their affinity for Ca^{2+}, are involved in tension development in mammalian skeletal muscle. For pCa < 6.50, the likely sites are the two low-affinity binding sites on troponin-C (Potter and Gergely, 1975). However, at still lower Ca^{2+} concentrations, the number and identity of additional Ca^{2+} binding sites can not be accurately predicted on the basis of the calculated Hill coefficient. The biphasic Hill plot of Fig. 3 *b* may very well be reflective of cooperativity among Ca^{2+} binding sites during the process of tension development (see Cornish-Bowden and Koshland, 1975, for a discussion of the effects of cooperativity on Hill plots). In fact, the steeper portion of the apparently biphasic Hill plot could represent a transitional region at intermediate Ca^{2+} concentrations

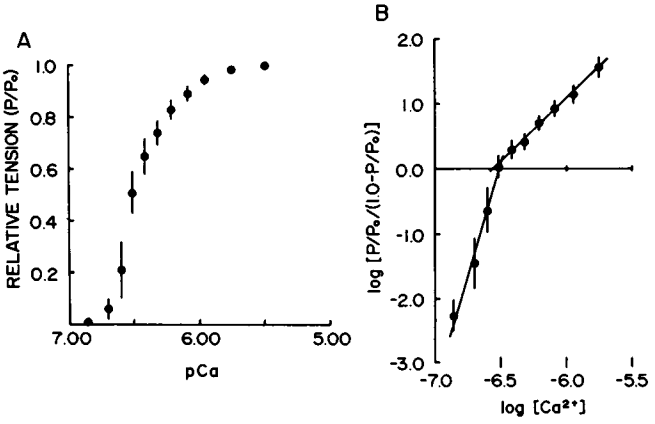

FIGURE 3 Plot of mean tension-pCa data obtained from 15 fiber segments at sarcomere lengths in the range 2.46–2.62 μm. In part *a*, relative tension (P/P_o) is plotted as a function of pCa; in part *b*, this same data has been used to construct a Hill plot. The error bars indicate ± 1 SD.

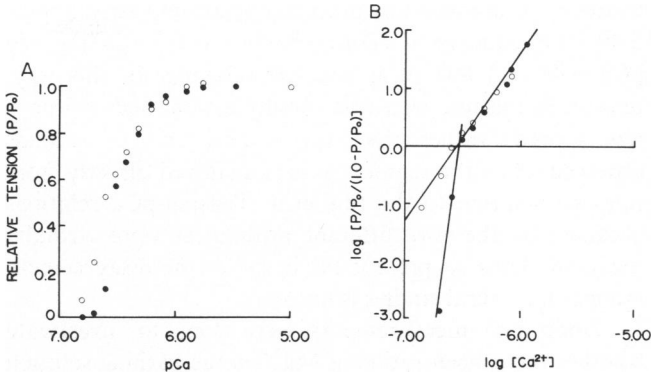

FIGURE 4 Plot of tension-pCa data obtained from one segment at optimum and stretched lengths. In *a*, relative tension (P/P_o) is plotted as a function of pCa at sarcomere lengths of 2.50 μm (•) and 3.24 μm (○). In *b*, this same data has been used to construct a Hill Plot. Segment no. 11382–2; overall length was 2.08 mm at optimum length. P_o, 1.48 kg/cm^2.

between asymptotes at high and low Ca^{2+} at which Ca^{2+} binding would occur at the sites of lowest and highest affinities, respectively. This latter interpretation is further complicated by the likely possibility that tension development cannot occur unless there is binding at both types of sites. Under this condition, it is improbable that a Hill plot in which the dependent variable is an expression of developed tension will resolve Ca^{2+} binding involving only the high affinity site(s). In this regard, Fuchs and Fox (1982) have in fact shown that in rabbit psoas muscle fibers the percent saturation of Ca^{2+} binding is greater than percent force over most of the range of activating Ca^{2+} concentrations.

Interestingly, increasing the sarcomere length to 3.00–3.25 μm resulted in a marked change in the tension-pCa relation (Fig. 4 *a*), with increases in tension at the lowest Ca^{2+} concentrations studied. At this length, the Hill plot of the data (Fig. 4 *b*) is described by a single straight line having a calculated slope (n) of 2.68 ± 0.38 (average of seven segments). Thus, one might speculate that at moderately stretched sarcomere lengths the activation of tension development involves only a single calcium binding step, and this presumably would be mediated via the low affinity Ca^{2+}-binding sites on TnC. The finding that tension development in stretched fibers was increased only at high pCa is in general agreement with Endo's (1972, 1973) results obtained in frog skeletal muscle fibers; however, this finding is inconsistent with previous results obtained by Moisescu and Thieleczek (1979) in frog muscle and Hibberd and Jewell (1982) in rat ventricular muscle. Such results may represent species and/or muscle type differences in the mechanism of tension development, though in light of Endo's result this does not seem likely at least for skeletal muscle. Another difference between these studies is the use of a rapid activation technique by Moisescu and Thieleczek and Hibberd and Jewell. To test whether this might account for the disparate results, the activation protocol for several fibers (n = 5) in the present study was altered so that each of the activations at low Ca^{2+} concen-

trations was immediately preceded by an activation at pCa 5.49. The sequence of solution baths was (*a*) relaxing, (*b*) pCa 5.49, (*c*) test pCa, and (*d*) relaxing. In this way, tension at the test pCa was rapidly established, within a few seconds rather than the several tens of seconds observed when the segment was transferred directly from relaxing solution to the test pCa. Tension-pCa relations obtained by the two different procedures were virtually identical. Thus, at present the basis for the disagreement among the several studies is unclear.

Additional measurements were done to investigate whether the concentration of Mg^{2+} in the bathing solution would influence the shape of the Hill plot. Because the high-affinity Ca^{2+} binding sites on TnC are probably occupied by Mg^{2+} at physiological [Mg^{2+}] (Robertson et al., 1981), it was conceivable that at the ~0.05 mM free Mg^{2+} used above, these sites would be unoccupied in relaxed muscle and would only bind Ca^{2+} when the segment was activated. Tension-pCa relations were obtained in fiber segments in activating solutions containing either 0.05 or 3.2 mM free Mg^{2+} (Fig. 5 *a*). Readily apparent is the rightward shift to higher Ca^{2+} concentrations of the relationship measured in the high Mg^{2+} solution, a phenomenon that has been reported previously (Donaldson and Kerrick, 1975). At sarcomere lengths in the range 2.46–2.62 μm, the Hill plots of the data (Fig. 5 *b*) at both high and low [Mg^{2+}] are best fit by two straight lines. The slopes of the respective lines are virtually identical in the two cases. Therefore, the biphasic nature of the Hill plot appears to be related specifically to the binding of Ca^{2+}. Activation of stretched fiber segments (n = 4) in high Mg^{2+} solutions yielded straight line Hill plots, similar to the results presented earlier (Fig. 4 *b*) for fibers in solutions containing our standard concentration of Mg^{2+}.

While at present the molecular basis for the length-dependent alteration in the form of the tension-pCa relation is unknown, there are several plausible explanations for this finding. The first of these involves a Ca^{2+} activation

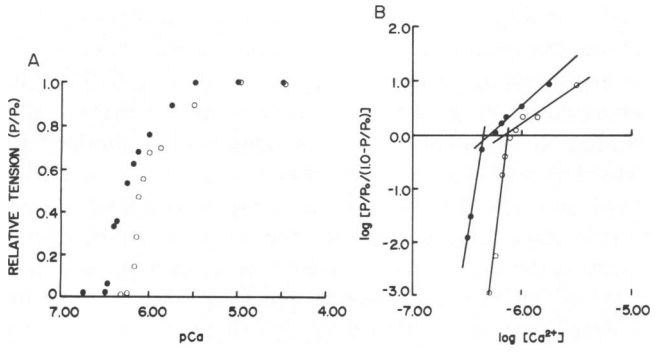

FIGURE 5 Plot of tension-pCa data, and Hill plot (*B*), obtained from one segment at low (•) and high (○) free Mg^{2+} concentrations. Segment no. 5382–2; overall length was 1.89 mm; sarcomere length was 2.49 μm; P_o was 1.49 kg/cm² at 0.05 mM free Mg^{2+} and 1.52 kg/cm² at 3.2 mM free Mg^{2+}.

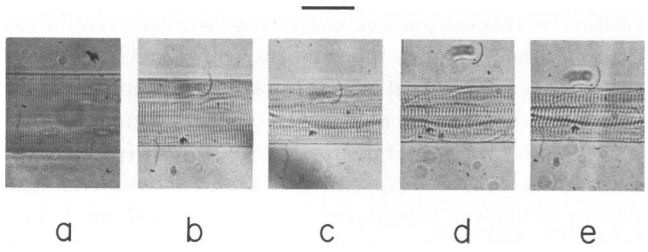

FIGURE 6 Light photomicrographs of a relaxed single fiber segment at five overall lengths. These photographs demonstrate the decreases in fiber diameter obtained when sarcomere length was increased: (*a*) *SL* = 2.52 μm, width = 76 μm; (*b*) 2.83 μm, 66 μm; (*c*) 3.10 μm, 58 μm; (*d*) 3.36 μm, 55 μm; (*e*) 3.61 μm, 51 μm. Segment #5482-1.

of the thick filament, in addition to that on the thin filament. Ca^{2+} binding to the thick filament could be involved in the activation of cross-bridges, perhaps by allowing them to swing out away from the thick filament to increase the likelihood of interaction with thin filament sites. A similar scheme was suggested by Endo (1973) who largely discounted this possibility when shrinkage of the fiber segment with high molecular weight polymers failed to increase the tension developed at optimum length and low levels of Ca^{2+}. However, Maughan and Godt (1981) have recently shown that shrinkage of fibers with high molecular weight polymers in the bathing solution substantially reduces the tension-generating capabilities of the fibers even at high Ca^{2+} levels. This result brings into question whether shrinkage induced in such a manner is comparable to the decrease in diameter observed when a fiber is stretched. To make a scheme involving Ca^{2+} activation of the thick filaments consistent with the present data (Fig. 3), the Ca^{2+} threshold for such an activation would have to be higher than that for the thin filaments. In this way, extension of the segments, which is accompanied by a substantial decrease in fiber diameter (Fig. 6), would preclude the need for cross-bridge activation, since the cross-bridge heads would be in close approximation to the thin filaments due to the decreased lateral spacing within the filament lattice. Thus, at stretched lengths, tension development would be regulated entirely by the thin filament–linked system. Validation of this hypothesis would require a demonstration that Ca^{2+} activation causes a movement of cross-bridge heads away from the thick filament backbone, a point that has yet to be clearly resolved (Haselgrove and Huxley, 1973; Matsubara and Yagi, 1980; Huxley, 1980), and also that myosin specifically binds Ca^{2+} with a relatively high affinity in the physiological concentration range for Ca^{2+} (see Bagshaw, 1980, for a review). With respect to the latter, Robertson et al. (1981) have modeled the time course of Ca^{2+} binding to troponin, myosin, and other Ca^{2+}-binding proteins of skeletal muscle and concluded that Ca^{2+} binding to myosin during contraction would be small at likely physiological Mg^{2+} levels. Certainly, this conclusion brings into question the physiological role of Ca^{2+} binding to myosin, though it must be remembered that these calculations are based on

binding constants determined using purified myosin in solution and thus may not apply to myosin in the intact fiber. For example, it has recently been shown that the apparent affinity of TnC for Ca^{2+} is decreased when the measurements are made using intact thin filaments (Potter and Zot, 1982).

A second possibility to explain our results is that binding of Ca^{2+} to sites on myosin would stabilize myosin in a configuration with an increased probability for interaction with the thin filament, a hypothesis modified from one that was suggested by Pemrick (1977). In this case also, the Ca^{2+} effect upon myosin might be bypassed by increasing the fiber length, thereby decreasing the lateral separation of the filaments. Pemrick showed that the actin-activated Mg-ATPase of myosin deficient in LC_2 light chain in fact has a Ca^{2+} sensitivity that is decreased relative to native myosin, though she interpreted her results in terms of an altered Ca^{2+} sensitivity of the regulatory proteins associated with the thin filament (see Bremel and Weber, 1972). In this regard, Godt and Maughan (1981) have suggested that changes in tension observed following compression of fibers using long-chain polymers are due to alterations in interfilament spacings, which in turn might be expected to alter the attachment-detachment rate constants of the cross-bridges at a given Ca^{2+} concentration. Alternatively, it is conceivable that phosphorylation of myosin LC_2, which is a Ca^{2+}-dependent phenomenon (Morgan et al., 1976) that has been observed to occur in tetanically stimulated skeletal muscle of the rabbit (Stull and High, 1977), is in some way involved in an activation of the thick filament. Further work is required to determine whether Ca^{2+}-activation of tension development involves an activation of the thick as well as the thin filaments.

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