

Extra Calcium on Shortening in Barnacle Muscle

Is the Decrease in Calcium Binding Related to Decreased Cross-Bridge Attachment, Force, or Length?

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ABSTRACT Barnacle single muscle fibers were microinjected with the calcium-specific photoprotein aequorin. We have previously shown (Ridgway, E. B., and A. M. Gordon, 1984, *Journal of General Physiology*, 83:75–104) that when barnacle fibers are stimulated under voltage clamp and length control and allowed to shorten during the declining phase of the calcium transient, extra myoplasmic calcium is observed. The time course of the extra calcium for shortening steps at different times during the calcium transient is intermediate between those of free calcium and muscle force. Furthermore, the amplitude increases with an increased stimulus, calcium transient, and force. Therefore, the extra calcium probably comes from the activating sites on the myofilaments, possibly as a result of changes in calcium binding by the activating sites. The change in calcium binding may be due, in turn, to the change in muscle length and/or muscle force and/or cross-bridge attachment per se. In the present article, we show that the amount of the extra calcium depends on the initial muscle length, declining at shorter lengths. This suggests length-dependent calcium binding. The relation between initial length and extra calcium, however, parallels that between initial length and peak active force. The ratio of extra calcium to active force is therefore virtually independent of initial length. These data do not distinguish between a direct effect of length on calcium binding and an indirect effect owing to changes in cross-bridge attachment and force through some geometrical factor. The amount of extra calcium increases with the size of the shortening step, tending toward saturation for steps of $\geq 10\%$. This experiment suggests that calcium binding depends on muscle force or cross-bridge attachment, not just length (if at all). There is much less extra calcium seen with shortening steps at high force when the high force results from stretch of the active muscle than when it results from

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increased stimulation of muscle. Thus, the number of attached cross-bridges appears to be more important than force itself. After a conditioning step decrease in length, force redevelops. A second test shortening step during force redevelopment produces less extra calcium than is seen in the absence of the conditioning step. Delaying the test step until more force has redeveloped increases the extra calcium seen with the test step, the increase paralleling the force redevelopment. This implies that calcium binding is increased by cross-bridge reattachment. These experiments do not exclude the existence of length-dependent calcium binding, but strongly suggest that there is increased calcium binding to the activating sites when cross-bridges attach.

INTRODUCTION

Activation of muscle contraction by calcium is well established (Ebashi and Endo, 1968). In many muscles, this occurs through the calcium binding to a component of the thin filament, troponin. This initiates a series of steps leading to the interaction of the myosin cross-bridge with actin, producing force. Calcium binding to troponin in myofibrils (Grabarek et al., 1983) shows a steep dependence upon free calcium, as does calcium activation of force in "skinned" fibers (Brandt et al., 1980) or of ATPase in myofibrils (Grabarek et al., 1983). One explanation for this steep relationship (proposed for isolated myofibrils by Bremel and Weber, 1972, and for intact fibers by Edman and Kiessling, 1971) is that calcium binding to the activating sites is increased by attached cross-bridges. This can also be inferred from the response of aequorin-injected barnacle single muscle fibers to length changes made during the declining phase of the calcium transient (Ridgway and Gordon, 1984). In this preparation, extra light (extra calcium) occurs in response to a step decrease in length and force. On the basis of its time course, dependence upon force and stimulation, and redeveloped force after the release, the extra light was attributed to calcium dissociating from the activating sites. The extra calcium appearing in the sarcoplasm in response to a shortening step (Gordon and Ridgway, 1978; Allen and Kurihara, 1982; Housmans et al., 1983; Stephenson and Wendt, 1984) could arise from the decrease in force and/or cross-bridge detachment and/or the length change itself.

The calcium sensitivity of a skinned muscle fiber is dependent upon sarcomere length, with sensitivity increasing with sarcomere length (Endo, 1972; Fabiato and Fabiato, 1978; Hibberd and Jewell, 1982). In recent reviews, Stephenson and Wendt (1984) have argued that the change in length itself affects calcium binding in skeletal and cardiac muscle, while Allen and Kentish (1985) have argued that changes in cross-bridge attachment and force were more important. This article describes experiments on the importance of length, force, and cross-bridge attachment on calcium binding to the activating sites. Our conclusions are that sarcomere length and muscle force may influence calcium binding under certain circumstances, but the most important determinant is cross-bridge attachment. Preliminary reports of this work have appeared (Gordon and Ridgway, 1985; Ridgway and Gordon, 1986).

METHODS

The methods used were similar to those described previously (Ridgway and Gordon, 1984). Single muscle fibers from the giant barnacle, *Balanus nubilus*, were dissected, cannulated, and microinjected with an aequorin solution. Native aequorin (Shimomura et al., 1962), which is a mixture of several related photoproteins (Blinks et al., 1976), and single purified molecular forms of aequorin (Shimomura, 1986) were used. The results were independent of the aequorin solution used (see Discussion). Single fibers were stimulated under voltage-clamp conditions using a double-spiral electrode inserted along the length of the fiber. Voltage-clamp conditions are required to avoid the marked effect of changes in initial muscle length on calcium release from the sarcoplasmic reticulum (SR) (Ridgway and Gordon, 1975; Gordon and Ridgway, 1976). Changes in calcium binding to the activating sites are inferred by observing calcium dissociated from the binding sites in response to a step change in muscle length. Therefore, it is important to keep SR calcium release constant. The initial length of the fiber was set so that there was just detectable passive tension (this was near the rest length in the animal). We did not measure a sarcomere length for each fiber, because barnacle fibers show a systematic variation of sarcomere length along their lengths (Gayton and Elliott, 1980). Muscle length was measured and controlled using an optical, electromechanical feedback circuit driving a shaker pot (V47/3, Ling Electronics, Royston, England) (see also Fig. 1 in Ridgway and Gordon, 1984). This allowed us to change the muscle length by 1–2 mm in <10 ms and regulate it at specified values. Force was measured using a transducer (DSC-6, Kistler-Morse, Bellevue, WA). Light from the injected aequorin was measured by a photomultiplier having a 2-in.-diam photocathode that was larger than the exposed fiber length and thus collected a fraction of the total light emission that was unaffected by changes in muscle length. The light signal was converted to a "calcium signal" by assuming that the aequorin light is related to free sarcoplasmic calcium through a 0.4 power [$Ca = K(\text{light})^{0.4}$] (see Blinks et al., 1982; Ridgway and Gordon, 1984). For these calculations, the total light intensity was used, including the resting glow. This procedure is identical to that used in our previous article (Ridgway and Gordon, 1984).

RESULTS

The Basic Effect

Fig. 1 illustrates the basic effects of a quick shortening step (release) on the aequorin light signal (calcium transient) and isometric force. A shortening step during the declining phase of the calcium transient causes extra light (Fig. 1, right), seen by comparing the falling phase of the calcium transient with the control contraction (Fig. 1, left). Subtracting the control calcium transient (Fig. 1, left) from the experimental record (Fig. 1, right) gives the magnitude and time course of the extra light (Fig. 1, "extra light"). The relative magnitude and time course of extra calcium can be calculated from it (extra calcium = $K[(\text{extra light} + \text{control light} + \text{resting glow})^{0.4} - (\text{control light} + \text{resting glow})^{0.4}]$) (see Ridgway and Gordon, 1984). The extra calcium is probably from the activating sites, recently identified in barnacles as troponin C (TnC) (Dubyak, 1985; Potter et al., 1986). The release of calcium from TnC is due to a decrease in its calcium-binding constant. The "extra calcium" is therefore due to a change in calcium binding induced by changes in length and/or force and/or cross-bridge attachment. Deciding which of these causes is most important is not simple, because

(as is obvious from Fig. 1) a length change also changes the force, and in addition permits a number of cross-bridges to break and re-form. Therefore, additional experiments were performed.

The Length-Force Relation and Extra Calcium

The coupling between muscle length and force is described by the length-active force relation. Variations in active force have been attributed to changes in the number of attached cross-bridges (Gordon et al., 1966), but also possibly to changes in calcium binding and activation (Allen and Kentish, 1985). Thus, changes in the extra light seen on shortening steps with variations in the initial muscle length seemed an important starting point for understanding whether length or force or cross-bridge attachment was determining calcium binding and the extra light seen with shortening steps. As in other muscles, the resting and the peak active forces depend upon the initial muscle length (Fig. 2). All fibers

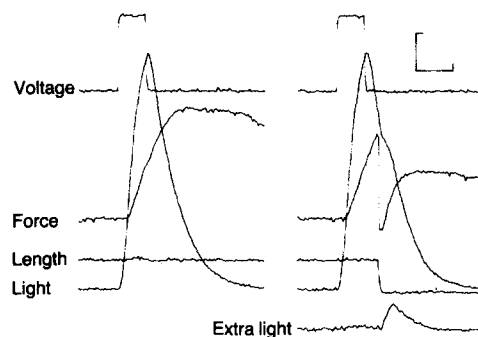


FIGURE 1. Effect of a shortening step made during the declining phase of the calcium transient. (*Left*) Control response. (*Right*) Response to a shortening step. Traces: membrane voltage, 20 mV/cal; isometric force, 3.3 g/cal; fiber length, 1.25 mm/cal; aequorin light signal (calcium transient), 500 nA/cal; extra light, 500 nA/cal. The extra light is computed by subtracting the control light signal (left) from the light signal in response to the shortening step (right). Horizontal sweep, 400 ms/cal; temperature, 9°C; fiber length, 23 mm; fiber weight, 49 mg; resting glow, 200 nA.

show some decline in peak active force at short muscle lengths, with little or no change at longer initial lengths over the small range investigated (although the total force, active plus passive, was always higher at longer lengths). Stimulation was set so that the maximum active force was always less than half of the maximum attainable level to prevent fiber damage owing to detachment from the cannula. Sarcomere lengths were not measured and they vary along the length of the fiber (Gayton and Elliott, 1980), so we cannot say precisely how much filament overlap there is and how much it changes as we increase or decrease muscle length. Muscle length was set to the value at which passive tension in the absence of the internal electrode is just detectable. For a given shortening step during the declining phase of the calcium transient, less extra light is seen when the fiber is released at the shorter than at the longer length (Fig. 3). The extra light is much smaller at the shorter muscle lengths, where the peak active force is smaller, and is relatively constant for releases at longer muscle

lengths, where the peak active force is relatively constant (Fig. 4). The extra light-length relation (Fig. 4) thus parallels the active force-length relation (Fig. 2). Fig. 4 further shows the ratio of the extra light (measured for the shortening step) to the peak active isometric force (at that length). This ratio is relatively constant for all fibers studied. Therefore, the extra light (calcium) correlates with active force (not the total force). If the extra calcium indicates the amount of calcium bound to the activating sites, these results are consistent with a model in which length affects calcium binding and determines cross-bridge attachment and thus active force. Alternatively, if cross-bridge attachment affects calcium binding, the parallel between calcium bound to the activating sites and active force could also be a result of cross-bridge-dependent calcium binding. We performed several experiments in which the extra light was measured under conditions of varying length, force, and cross-bridge attachment in order to distinguish between the two hypotheses.

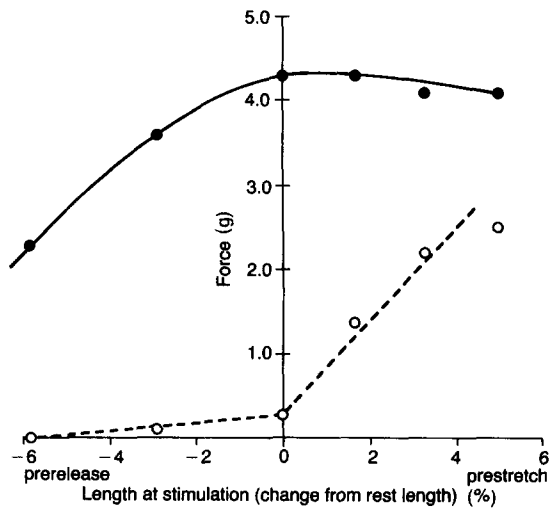


FIGURE 2. Length-force relationship for a barnacle single muscle fiber. The peak active force (filled circles) is the increase upon stimulation above the steady passive force at that muscle length. The passive force (open circles) is the increase in the steady level at the new length above that of the shortest initial lengths. The muscle fiber was prestretched or prereleased by the indicated percent of the rest length immediately preceding the stimulus. The lines are a visual fit to the data. Fiber length, 24 mm; fiber weight, 78 mg; resting glow, 500 nA.

The Extra Light (Calcium) Tends toward Saturation with Increasing Length Step

If the extra calcium on shortening results from a length-dependent calcium-binding mechanism, additional light should be seen as the amplitude of the length step increases. If calcium binding depends upon cross-bridge attachment, however, rapid length changes large enough to detach all the cross-bridges should produce the maximum extra calcium (light). Further shortening should not produce any additional extra light except as cross-bridges reattach and detach or remain weakly bound during muscle shortening. We therefore investigated the dependence of the amount of extra light upon the amplitude of the release step. The relation between shortening and lengthening steps is such that extra calcium appears on shortening, but a decrease in calcium, below control, occurs on lengthening, which implies an apparent absorption of calcium (Ridgway and Gordon, 1984). In our previous article (Ridgway and Gordon, 1984), the

amount of extra light with the shortening step did not appear to saturate, but our length changes were restricted to <10% of the initial length. The results with larger length changes are shown in Fig. 5. The amount of extra light increases as the amplitude of the shortening step is increased, tending toward saturation for steps above 10%. Fig. 5A shows the extra light (as a percentage of the maximum) for a single fiber at two stimulus intensities and force amplitudes. Fig. 5B shows the extra calcium for four different fibers in response to length changes. The fibers shown redeveloped some force even after the largest length changes and were able to shorten to the new length. The tendency toward saturation of the amount of extra calcium with increasing amplitude of the

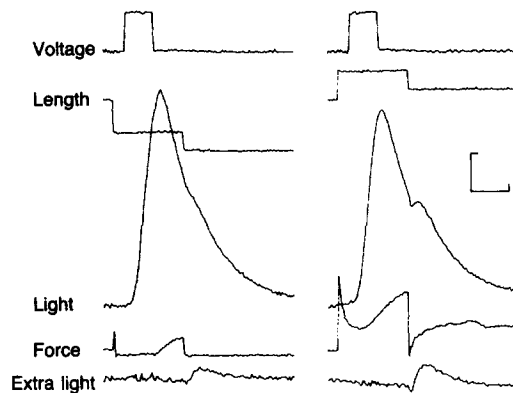


FIGURE 3. Experimental records demonstrating the effect of the muscle length at the time of stimulation on the extra light seen after a shortening step. The data are from the fiber shown in Fig. 2. Traces: membrane voltage, 30 mV/cal; fiber length, 1.5 mm/cal; aequorin light signal (calcium), 1,000 nA/cal; isometric force, 3.4 g/cal; extra light (calcium), 1,000 nA/cal. The extra light is that seen after the shortening step during the declining phase of the calcium transient obtained by subtracting the luminescence seen during a control record with the same stimulus but no length change

from that seen with the length change, such as is shown in the light trace (see Fig. 1). The record on the left shows a prerelease (shortening) of 5% of the fiber length; the record on the right shows a prestretch (lengthening) of 5.8% of the fiber length. The same shortening step of 3% was imposed at the same time during the declining phase of the calcium transient for both records. Notice the decrease in the passive and peak active force at the shorter initial fiber length at the time of stimulation, the increase in the passive and peak active force at the longer length, and the increase in the extra light for the same shortening step at the longer initial length. Horizontal sweep, 400 ms/cal; temperature, 7°C.

shortening step would be expected if calcium binding were influenced by cross-bridge attachment and all cross-bridges could be detached by a large enough shortening step. However, the length changes are large (sometimes 10–20%), far greater than required to reduce the active force to zero during the shortening step, and the extra light did not always saturate. Therefore, one cannot rule out some effect of large changes (>20%) of muscle length on calcium binding (Endo, 1972; Fabiato and Fabiato, 1978), although the short-range role of cross-bridges clearly dominates in these experiments.

If the rapid shortening step is followed a short time later by a restretch to the initial length, the amount of extra light is virtually the same as if the fiber had

not been restretched (Fig. 6). Only when the restretch is so close to the release as to truncate the shortening step did the peak of the extra light decrease to any appreciable extent. In some cases, the area of the extra light was decreased by a small amount (25% in this figure), but far less than would be expected if the restretch totally reversed the effects of shortening on the calcium affinities. To estimate this, one can measure the area of the extra light seen with the shortening step delivered alone, but at the time in the calcium transient at which the restretch occurred. For the fiber in Fig. 6, this gives an extra light area that is >70% of the area of the light from the original step. The expected reduction in the area of the extra light by the restretch would be 70% if calcium binding is length dependent rather than the mere 25% observed in Fig. 6. Thus, even though the fiber was returned to its initial length (so that no net length change

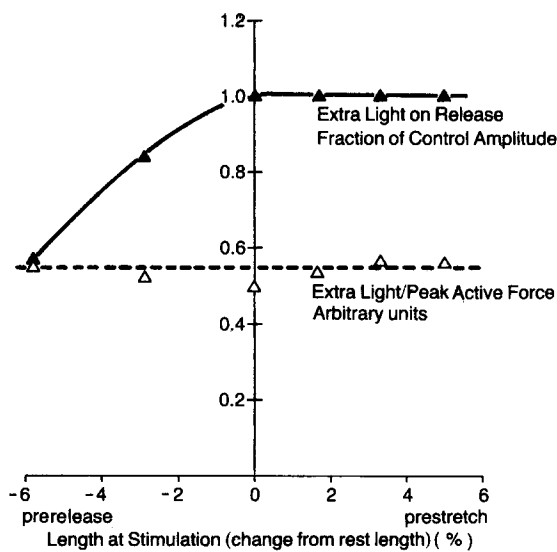


FIGURE 4. Plot of the amplitude of the extra light (filled triangles) seen with a 3% shortening step from different initial fiber lengths as a function of the initial length. The data are from records such as those shown in Fig. 3. The extra light is expressed as a fraction of the control amplitude seen for the 3% shortening step from the control (rest length). Notice that the dependence of the extra light on the initial length parallels that of the peak active force (Fig. 2). Plotting the ratio of the extra light to peak active force (open triangles) as a function of the initial length at the time of stimulation shows that the ratio is independent of the initial length. The lines are a visual fit to the data.

occurred), virtually the same amount of extra light was seen. This suggests that the calcium bound depends on cross-bridge attachment, not on absolute muscle length.

Finally, the amount of extra calcium depends not only on the extent of the shortening step but also on the rate of shortening (Fig. 7). When the rate of shortening decreases below the maximum rate of active fiber shortening, the amount of extra calcium decreases. Because of the limitations of the present mechanical setup, the higher shortening rates needed to explore rapid cross-bridge transients (Ford et al., 1977) were not investigated. One might argue that these data do not contradict strict length dependence since, for slower releases, the fiber length is still being changed late in activation when the free calcium (and possibly the bound calcium) is decreasing. However, for these experiments,

the rate of shortening, not the time taken to shorten, appears to be the crucial variable since, as shown in Fig. 7, the extra calcium for a given shortening value (as a fraction of the maximum for the total shortening) is the same for shortening ramps of 0.5 and 1.0 mm, even though the time taken to shorten 1.0 mm is

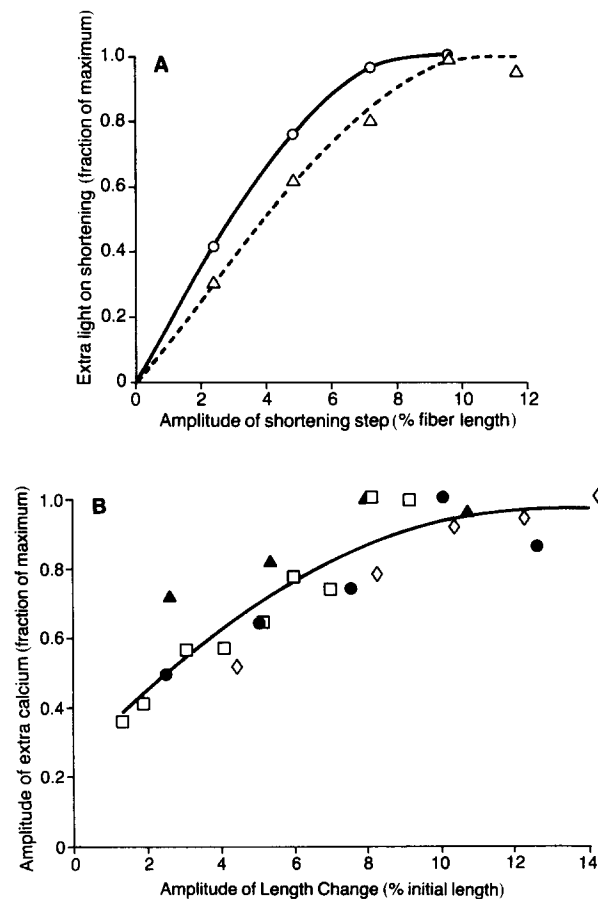


FIGURE 5. Plot of the amplitude of the extra light (*A*) or extra calcium (*B*) as a fraction of the maximum amplitude seen with a shortening step during the declining phase of the calcium transient (such as in Fig. 1) plotted as a function of the amplitude of the shortening step (as a percentage of the initial fiber length). (*A*) Data from one fiber at two stimulus intensities and peak active forces (triangles, 9.5 g; circles, 5.7 g). Fiber weight, 60 mg; fiber length, 22 mm. (*B*) Data from four fibers, each represented by a different symbol. Notice that both the peak amplitude of the extra light and extra calcium seen after the shortening step tends toward saturation as the amplitude of the shortening step is increased. The lines are the least-square fits of a second-order polynomial to the data.

twice as great. Thus, these data on the rate dependence of extra light also suggest that the length change per se is not the only critical factor in determining the amount of the extra light.

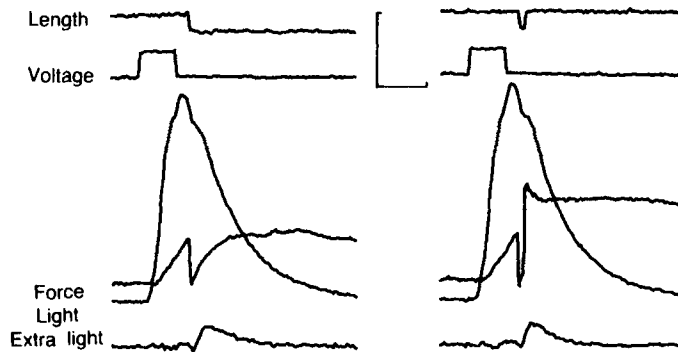


FIGURE 6. Experimental records demonstrating that restretching the fiber shortly after the shortening step does not affect the extra light to any appreciable extent. Traces: membrane voltage, 80 mV/cal; fiber length, 2 mm/cal; aequorin light signal (calcium), 200 nA/cal; isometric force, 4.6 g/cal; extra light (calcium), 200 nA/cal. The extra light is obtained by subtracting the luminescence seen during a control record, with the same stimulus but no length change, from that seen with the length change. In the record on the left, the fiber was shortened by 2.1% of the fiber length. In the record on the right, the fiber was shortened by the same amount as in the record on the left, but restretched 50 ms later. Note that the extra light trace has virtually the same amplitude in both records. The area of the extra light in this example on the right is a little (25%) smaller, but much larger than it would be if the restretch had totally reversed the extra light (see text for calculation). Horizontal sweep, 400 ms/cal; temperature, 7.2°C; fiber length, 24 mm; fiber weight, 78 mg; resting glow, 380 nA.

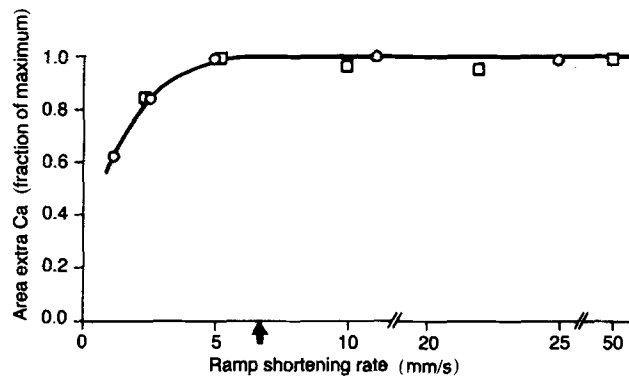


FIGURE 7. Effect of the rate of shortening on the area of the peak corresponding to extra calcium. Ramp releases of 0.5 mm (circles) total amplitude (2.2% of the fiber length) or 1.0 mm (squares) (4.3% of the fiber length) were initiated at a given time during the declining phase of the calcium transient. The area of the extra calcium, as a fraction of the maximum area, produced by a steady shortening ramp is plotted as a function of the rate of shortening during the ramp. The estimated maximum unloaded shortening velocity (6.7 mm/s, 0.29 muscle lengths/s) for this fiber is indicated by the arrow. Temperature, 8°C.

Extra Calcium Is Increased by Greater Muscle Force through Stimulation but not through Stretch

The extra calcium was measured under conditions whereby force was measured in two ways: by increasing the stimulus intensity, thereby increasing calcium activation, and by stretching the active muscle to a higher force, thereby increas-

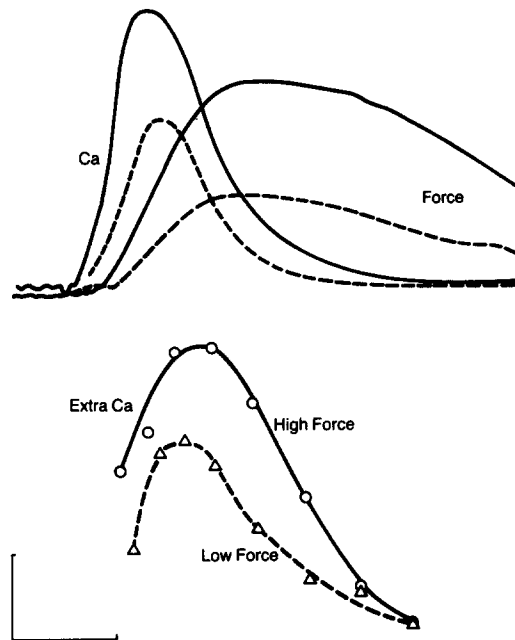


FIGURE 8. Extra calcium seen after a shortening step made at different times during the calcium transient for stimuli of two intensities producing the calcium transients and forces shown. (*Top*) calcium transient, relative units; isometric force, 4.5 g/cal. (*Bottom*) extra calcium observed with the shortening step of 5% of the initial fiber length imposed at different times during the calcium transient plotted as occurring at the time of the shortening step. The solid lines are used for the high-force calcium transient, force, and extra calcium (circles). The dashed lines are used for the low-force records. Calcium and extra calcium were calculated as in Ridgway and Gordon (1984). The data were derived as in Ridgway and Gordon (1984, Figs. 13 and 14). Horizontal sweep, 400 ms/cal; fiber weight, 38 mg; fiber length, 20 mm; temperature, 9°C. Note that at this slightly higher temperature, the calcium transient is faster than at the lower temperatures. Also, there is some variation between individual fibers, presumably representing differences in the SR, the amount of intracellular calcium binding proteins, ATP levels, intracellular pH, etc.

ing force in each cross-bridge at a constant calcium activation. Increased depolarization produces an increased calcium transient, increased calcium binding, increased force, and increased extra light for a given amplitude shortening step (Ridgway and Gordon, 1984, Fig. 10). Fig. 8 shows the amplitude of the calcium transient and force for two levels of depolarization (top) and the amount of the extra calcium seen for length changes made at different times during the calcium

transient (bottom). The extra calcium observed for the step change in length is less for the smaller-amplitude calcium transient, stimulus, and force. Since more calcium is bound with increased stimulus intensities, more extra calcium would be expected whether calcium binding depended upon force or length. Thus, the increased extra calcium is consistent with either explanation.

Stretching the fiber during activation produces both higher passive forces and higher active forces (Edman et al., 1978). The higher active force per cross-bridge (Edman et al., 1978; Julian and Morgan, 1979) might be expected to affect calcium binding if calcium binding to the activating sites were dependent on muscle force. Fig. 9 shows the extra light seen for a shortening step during

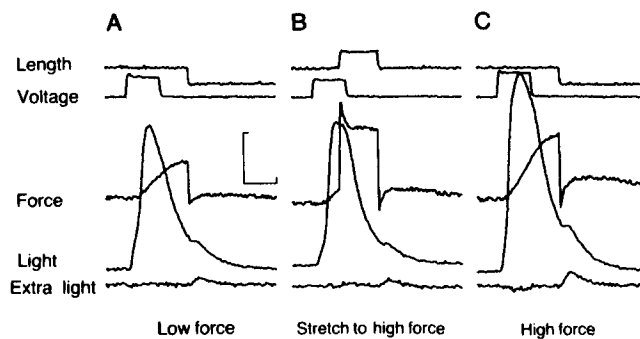


FIGURE 9. Effect of increased force on the extra light seen on shortening. Traces: fiber length, 2 mm/cal; membrane voltage, 40 mV/cal; isometric force, 4.5 g/cal; aequorin light signal (calcium), 400 nA/cal, and extra light (extra calcium) (seen above the control record with no length change), 400 nA/cal; plotted for three conditions. (A) Low stimulus, producing a low peak force. (B) Similar stimulus as in A but the fiber was stretched by 2.5% during contraction to a higher force. (C) Higher stimulus, producing a higher calcium transient and higher force comparable to that seen during the stretch in B. The shortening step in all records was 2.5% of the fiber length imposed at the same time during the declining phase of the calcium transient. Note the increased extra light when more cross-bridges are attached because of the greater stimulus intensity and resulting higher calcium transient. Horizontal time scale, 400 ms/cal; fiber weight, 38 mg; fiber length, 20 mm; temperature, 9°C; resting glow, 300 nA.

the declining phase of the calcium transient (A), after a stretch to a high force immediately preceding the release (B), and after the stimulus was increased to produce an active force equivalent to that achieved by the stretch (C). There is very little effect of stretch on the amplitude of the light seen with subsequent release, but a large increase when the same force was achieved by additional stimulation. Fig. 10 shows that these relationships hold for the extra calcium observed for length steps at different times during the declining phase of the calcium transient. In this fiber, stretching to obtain higher forces results in approximately the same extra calcium as in the control case, but, in many other fibers, stretching resulted in slightly less extra calcium. Increased force per cross-

bridge thus has little effect on the amplitude of the extra calcium and appears to increase the bound calcium only slightly, if at all.

The Extra Calcium Depends upon the Redeveloped Force

To test whether the extra calcium depends on force at a constant calcium activation or on length, force (or cross-bridge attachment) in the fiber during stimulation was varied by allowing the fiber to shorten. When a fiber shortens to the test length during contraction, force falls and subsequently redevelops. A second shortening step during force redevelopment can be used to probe bound

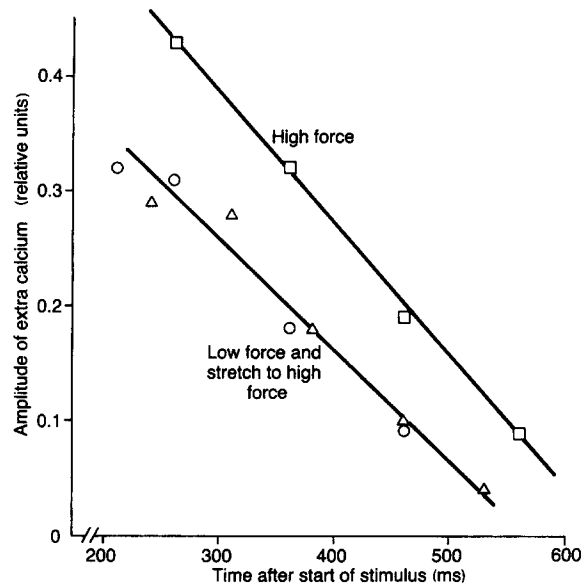


FIGURE 10. Amplitude of the extra calcium for shortening steps at different times during the declining phase of the calcium transient for the three conditions of low force (triangles), stretch to high force (squares), and stimulation to high force (circles), as illustrated in Fig. 9. The amplitude of the extra calcium is plotted against the time of the shortening step. In all cases, the shortening step was 3.5% of the fiber length. Note that, at all times, more extra calcium is seen when there is higher force through more calcium bound and more attached cross-bridges and no more extra calcium is seen when the higher force is attained through stretch at the same stimulus. The data are from same fiber shown in Fig. 9.

calcium during the force recovery. This two-step protocol is illustrated in Fig. 11. The first shortening, the conditioning step, resulted in a drop in force and a burst of extra light. A test step made during force redevelopment produced another drop in force and additional extra light. In order to determine how much the extra calcium on the test step was affected by the conditioning step, the amount of extra light observed for both the conditioning and test steps was compared with the light from a contraction at the test length with the same stimulus in which there was only the test step. Fig. 12 shows the length change protocols used to determine the amount of extra light owing to the test step

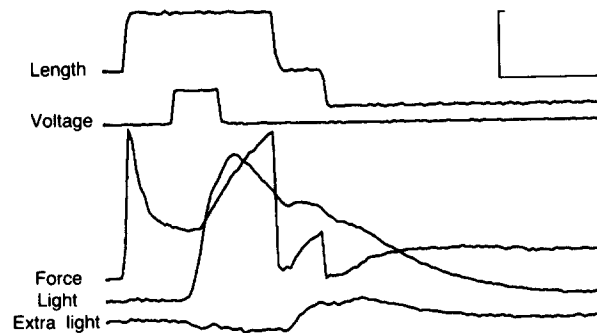


FIGURE 11. Experimental record illustrating the effect of the redeveloped force on the extra light seen with the shortening step. The extra light is seen accompanying the two shortening steps during the declining phase of the calcium transient. The first step is the conditioning step, dropping the force nearly to zero. The force does not fully return to the initial level because the length change is slow enough to allow some redevelopment of force during the length step. The second step occurs during the force redevelopment from the first and drops force to below the initial level. Fiber length, 2.5 mm/cal; membrane voltage, 40 mV/cal; isometric force, 3.7 g/cal; aequorin light signal (calcium), 1,000 nA/cal; extra light (calcium), 1,000 nA/cal. Notice the double-humped extra light seen accompanying the two length steps (of 15 and 10% of the fiber length) during the declining phase of the calcium transient. Horizontal sweep, 400 ms/cal; fiber weight, 40 mg; fiber length, 15 mm; temperature, 7°C; resting glow, 80 nA.

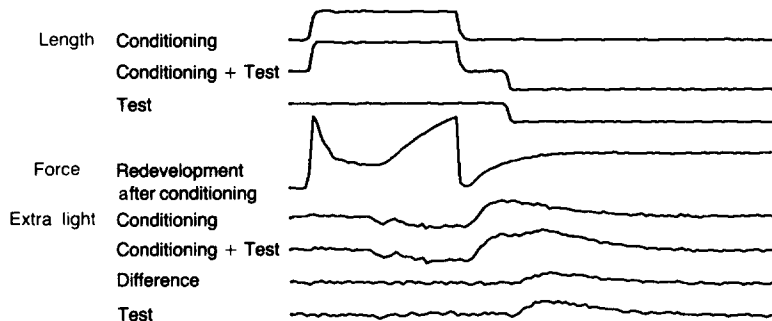


FIGURE 12. Illustration of the procedure used to determine the extra light seen with the test step when it is preceded by the conditioning step and when it is not. Above are the length traces illustrating the protocol with the conditioning step alone, and conditioning plus test steps together with a delay in between and with the test step alone; the force trace showing the response to the conditioning step alone, the fall, and subsequent redevelopment of force; and the extra light seen accompanying the various step protocols, the conditioning step alone, conditioning plus test steps together, the subtraction of the extra light for the conditioning step alone from that seen with conditioning and test steps together ("difference"), and finally that seen with test step alone. Note that more extra light is seen with the test step alone than when it is preceded by the conditioning step (compare "test" and "difference"). Thus, during the redevelopment of force after the conditioning step, less extra light is seen than if there had not been a conditioning step and a fall in force. The initial prestretch preceding the stimulation was performed so that the test step would always be initiated from the same fiber length whether it was preceded by the conditioning step or not.

alone. In each case, the extra light signal (the light signal with, minus that without, the length changes) is shown. The extra light of the conditioning step was subtracted from the extra light produced by the conditioning and test steps given together. The result is smaller than the extra light from the test step alone. Thus, less extra calcium is seen for a given shortening step immediately preceded by another step. When the timing of the conditioning step was held constant and the delay between the steps was increased (by changing the time of the test step), the effect of the conditioning step on the test step decreased. Fig. 13 shows the extra light seen on the test step as a function of the delay between the two steps. At longer intervals, the amplitude of the extra light recovered to what it would

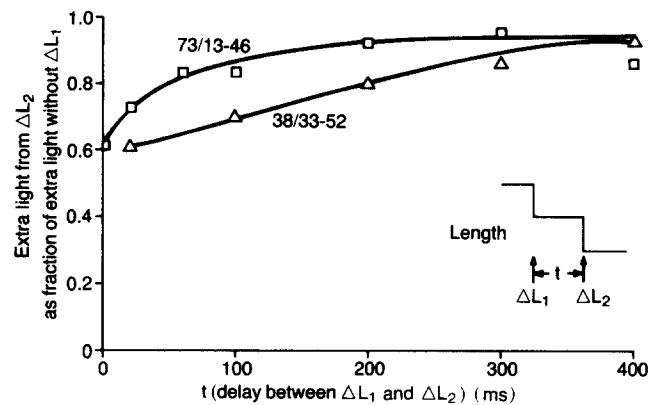


FIGURE 13. Recovery of the extra light seen with test step ΔL_2 after the conditioning step ΔL_1 , as a function of the time t between the two length steps, allowing redevelopment of force after the conditioning step. Extra light seen on the test step, ΔL_2 , is expressed as a fraction of the amplitude of the extra light seen when the test step, ΔL_2 , is preceded by the conditioning step, ΔL_1 , to what is seen when there is no conditioning step, ΔL_1 . Data from two fibers showing the slowest and fastest recovery of extra light. Note that the extra light is decreased only $\sim 40\%$ when the test step, ΔL_2 , is close to the conditioning step, ΔL_1 . The recovery of the extra light back to the control value parallels the redevelopment of force (see Fig. 14). For record 73/13-46, ΔL_1 was 3% and ΔL_2 was 3.5% of the fiber length. For record 38/33-52, ΔL_1 was 15% and ΔL_2 was 10% of the fiber length.

have been without the conditioning step. The time course of this recovery varied somewhat between fibers. For the shortest delays between the two steps, the extra light seen on the test step was $\sim 60\%$ of that seen without the conditioning step. Fig. 14 shows the redeveloped force after the conditioning step and the extra light on the test step relative to that from the conditioning step alone, scaled so that the time courses can be compared. The two fibers are those shown in Fig. 13. The amount of extra light on the test step recovered with about the same time course as did the force, lagging slightly behind force redevelopment in one (Fig. 14, top) and leading it slightly in the other (Fig. 14, bottom).

When the timing of the test step was kept constant and the delay between the steps was varied by initiating the conditioning step earlier, the same result was observed. The amount of extra light seen from the test step was always less than with no conditioning step and the recovery had approximately the same time course as the redevelopment of force after the step. In all of the cases above, the extra calcium was measured using a length change from the same initial length and under the same stimulating conditions. The results suggest that with these other conditions constant, the extra calcium depends strongly on the reattached cross-bridges and the force generation during force redevelopment.



FIGURE 14. Time course of the recovery of the extra light compared with the redevelopment of force after a conditioning shortening step for the two fibers shown in Fig. 13. The solid line is the force and the symbols are the recovery of the extra light. To facilitate comparison, the recovery of the extra light was scaled to have the same maximum as the redeveloped force and to start at the same minimum. Note that the recovery of the extra light parallels the redevelopment of force. Trace length, 2 s; temperature: upper trace, 7°C; lower trace, 7.5°C.

DISCUSSION

Our results show that while there may be a muscle length dependence, there is a strong effect of cross-bridge attachment and force production on calcium binding. The level of force per se is not a major factor, because stretching to higher forces (presumably increasing the force per cross-bridge, but not necessarily the number attached [Julian and Morgan, 1979]) does not increase the amount of extra calcium (see Figs. 9 and 10). Increasing force per cross-bridge does increase calcium binding to a slight extent (see Fig. 3 of Ridgway and Gordon, 1984), but this effect is small compared with the effect of decreased cross-bridge attachment. Attaching of cross-bridges during force redevelopment does, moreover, increase the amplitude of the extra light signal (Fig. 14). The data are thus consistent with a model in which attached cross-bridges cause increased calcium binding; they do not exclude an effect of muscle length per se (Fig. 4). Our experiments do, however, rule out length change per se as being entirely responsible for the extra light on shortening (suggested by Stephenson and Wendt, 1984). Specifically, the tendency toward saturation of the extra light

signal with length changes (Fig. 5), the dependence of extra light on the velocity of the shortening step (Fig. 7), the lack of an effect of the duration of the shortening step on the extra light (Fig. 6), and the dependence of the extra light signal on the delay between the two shortening steps rather than only on their amplitude (Fig. 14) all argue against the proposition that length is the predominant determinant of calcium binding.

If length is not the predominant determinant of calcium binding, then how can the results of the force-length relationship shown (Figs. 2 and 4) be explained? One interpretation of the results shown in Fig. 4 is that there is an interaction between cross-bridge attachment and calcium binding such that they are linked in a positively cooperative manner; calcium binding allows cross-bridge attachment, but the number of attached cross-bridges, in turn, determines calcium binding. These experiments provide support for the notion that calcium binding, which may be cooperatively linked to cross-bridge attachment, is a substantial factor in determining the force-length relationship (particularly at short sarcomere lengths) (discussed by Allen and Kentish, 1985).

Positive cooperativity between cross-bridge attachment and calcium binding would steepen the relationship between muscle force and free calcium. Other factors, such as calcium binding to another site (Moss et al., 1983) or cross-bridge kinetics depending on force through the myofilament lattice strain (Brandt et al., 1980), may contribute to this relationship, which is much too steep to be accounted for by a straightforward calcium binding to sites on troponin. Our data support the hypothesis (Hill et al., 1980) that at least two factors are involved in this cooperativity. One factor is calcium binding to troponin, which provides a long-range cooperativity through tropomyosin and actin activating the thin filament (Grabarek et al., 1983) (possibly the whole filament: see Brandt et al., 1984, but see also Walsh et al., 1984; Moss et al., 1986). Recent studies by Moss et al. (1985, 1986) suggest that removing some troponin units both brings about partial activation and increases the apparent calcium sensitivity of neighboring units, while removing TnC does the opposite. All these studies underline the role of tropomyosin in the cooperativity. The other factor is cross-bridge attachment, which (*a*) increases calcium binding to troponin, and (*b*) increases the probability of further cross-bridge attachment, both of which provide a strong local cooperativity. Our results support the hypothesis of cross-bridge-dependent calcium binding and thus suggest this as at least a partial explanation of the steep relationship. Our interpretation is consistent with the results and hypotheses of Edman and Kiessling (1971) and Bremel and Weber (1972) and with the fluorescent probe studies of Grabarek et al. (1983). It is also consistent with calcium-binding measurements (Fuchs, 1985; Hofmann and Fuchs, 1986) because muscle force per se is much less important than cross-bridge attachment.

A number of important controls have been done. The fibers were voltage-clamped to remove a possible length-dependent calcium release from the SR. The extra light was observed with injections of single pure isoforms (A, C, and D) of aequorin (Shimomura, 1986) as well as with whole native aequorin. This control rules out the possibility that the extra light is due to a small fraction (i.e., one or two isoforms) of native aequorin responding to the length change while the rest does not. Our previous article (Ridgway and Gordon, 1984) included

controls for the effects of the length change per se bringing about extra light through damage or other artifacts. The results of others on both intact and "skinned" muscle fibers (Allen and Kurihara, 1982; Housmans et al., 1983; Stephenson and Wendt, 1984) support the observation of extra light on release seen here and indicate that the phenomenon is not restricted to barnacle single muscle fibers but occurs in mammalian cardiac and skeletal muscle as well.

The calcium regulatory system in barnacle muscle has not been described fully. Lehman and Szent-Gyorgyi (1975) concluded that barnacle muscle has a dual regulatory system on both thin and thick filaments. Dubyak (1985) suggested that barnacle has only a single thin-filament regulatory system since myosin from barnacle muscle is not regulated by calcium in the presence of rabbit actin. Potter et al. (1986) have isolated barnacle troponin. Thus, the regulatory system is presumably through thin-filament troponin, as in mammalian striated muscle.

A specific molecular mechanism by which cross-bridge attachment increases calcium binding to TnC has not been worked out. Bremel and Weber (1972) showed this result for rigor bonds in rabbit myofibrils. Rigor bonds increased calcium binding, presumably to the calcium-specific sites on TnC. From detailed balance considerations, one would expect that as the calcium binding to TnC affects the actomyosin interaction, that actomyosin interaction would affect calcium binding (see Taylor, 1979). The enhanced binding presumably occurs because of a decreased off rate for calcium dissociation from the activating sites, as was measured by Rosenfeld and Taylor (1985) using S-1 rigor interactions with regulated thin filaments, and can be inferred from our observed decreased rate of decline of the extra calcium at higher forces (Fig. 8, bottom). Data from rabbit psoas muscle fibers containing rhodamine-labeled TnC (Yates et al., 1985) also imply that both calcium binding to TnC and cross-bridge attachment affect the orientation of the labeled TnC on the thin filament. Effects of cross-bridge attachment to actin may be transmitted to troponin through tropomyosin interactions along the thin filament (Hill et al., 1983; Moss et al., 1986). The specific interactions within the troponin complex that give rise to this cross-bridge-dependent effect await detailed analysis (see Leavis and Gergely, 1984). Calcium binding to the calcium-specific sites in isolated TnC becomes stronger when TnI binds to TnC, and then weaker when the TnC-TnI-TnT complex is reincorporated into a regulated thin filament (Grabarek et al., 1983). Calcium binding to TnC strengthens TnC binding to TnI and weakens the binding of the TnC-TnI complex to the thin filament (Leavis and Gergely, 1984). Perhaps cross-bridge attachment weakens the binding of TnC-TnI to the thin filament and shifts the calcium affinity of the calcium-specific sites on TnC back toward their higher-affinity state. More definitive measurements of calcium binding, possibly using the fluorescent probe labels, will shed light on this question (Johnson et al., 1978; Grabarek et al., 1983; Yates et al., 1985; Guth et al., 1986).

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