Stretch of Active Muscle during the Declining Phase of the Calcium Transient Produces Biphasic Changes in Calcium Binding to the Activating Sites

A. M. GORDON and E. B. RIDGWAY

From the Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195; and Department of Physiology and Biophysics, Medical College of Virginia, Richmond, Virginia 23298

ABSTRACT In voltage-clamped barnacle single muscle fibers, muscle shortening during the declining phase of the calcium transient increases myoplasmic calcium. This extra calcium is probably released from the activating sites by a change in affinity when cross-bridges break (Gordon, A. M., and E. B. Ridgway. 1987. J. Gen. Physiol. 90:321–340). Stretching the muscle at similar times causes a more complex response, a rapid increase in intracellular calcium followed by a transient decrease. The amplitudes of both phases increase with the rate and amplitude of stretch. The rapid increase, however, appears only when the muscle is stretched more than ~0.4%. This is above the length change that produces the breakpoint in the force record during a ramp stretch. This positive phase in response to large stretches is similar to that seen on equivalent shortening at the same point in the contraction. For stretches at different times during the calcium transient, the peak amplitude of the positive phase has a time course that is delayed relative to the calcium transient, while the peak decrease during the negative phase has an earlier time course that is more similar to the calcium transient. The amplitudes of both phases increase with increasing strength of stimulation and consequent force. When the initial muscle length is increased, both phases of the response to stretch increase in proportion to the active force. A large decrease in length (which drops the active force to zero) decreases the extra calcium seen on a subsequent restretch. After such a shortening step, the extra calcium on stretch recovers (50 ms half time) toward the control level with the same time course as the redeveloped force. Conversely, stretching an active fiber decreases the extra calcium on a subsequent shortening step that is imposed shortly afterward. Enhanced calcium binding due to increased length alone cannot explain our data. We hypothesize that the calcium affinity of the activating sites increases with cross-bridge attachment and further with cross-bridge strain. This accounts for the biphasic response to stretch as follows: cross-bridges detached by stretch first decrease calcium affinity, then upon reattachment increase...
calcium affinity due to the strained configuration brought on by the stretch. The experiments suggest that cross-bridge attachment and strain can modify calcium binding to the activating sites in intact muscle.

**Introduction**

Calcium binding to the thin filament activates contraction in many striated muscle fibers (Ebashi and Endo, 1968). Experiments on isolated proteins and on skinned muscle fibers in which direct calcium binding (Bremel and Weber, 1972; Fuchs, 1977) or structural changes in one of the troponin subunits were measured (Trybus and Taylor, 1980; Greene, 1986; Guth and Potter, 1987; Schulte et al., 1987) suggest that cross-bridge attachment increases calcium binding to these sites. There is considerable evidence that this occurs in intact muscle fibers. When shortening steps are used to reduce the number of attached cross-bridges, the concentration of myoplasmic free calcium increases in barnacle muscle (Gordon and Ridgway, 1978, 1987; Ridgway and Gordon, 1984), in intact cardiac muscle (Allen and Kurihara, 1982; Housmans et al., 1983), and in skinned skeletal and cardiac muscle (Stephenson and Wendt, 1984; Allen and Kentish, 1988). Reducing the number of attached cross-bridges decreases calcium binding to the thin filament activating sites, suggesting that the converse may be true. Although some of the changes in calcium binding may be due to concomitant changes in sarcomere length (Endo, 1972), the major effect is due to the change in the number of cross-bridges attached (Gordon and Ridgway, 1987; Allen and Kentish, 1988). Calcium binding to troponin may be sensitive to cross-bridge attachment per se, or to cross-bridge strain or the biochemical state of the cross-bridge (e.g., the presence of bound nucleotide and phosphate). Results with skinned muscle fibers in which the endogenous troponin-C (TnC) has been replaced with exogenous fluorescently labeled TnC imply that cross-bridge states can affect the TnC structure as measured by the fluorescent probe (Guth and Potter, 1987; Gordon et al., 1988). Although effects of cross-bridge strain on calcium binding have not been demonstrated, strain has been shown to affect rate constants in the cross-bridge cycle (Goldman et al., 1984; Webb et al., 1986).

The present studies were undertaken to determine if cross-bridge strain produced by stretch increases calcium binding to the thin filament activating sites in intact muscle fibers. Griffiths et al. (1980) suggested that large stretches of frog single muscle fibers during activation produce cross-bridge detachment and rapid reattachment. The stretch-induced detachment–reattachment cycle should affect the calcium bound to the thin filament activating sites (troponin in barnacle muscle; Potter et al., 1986) if cross-bridge strain is important. Stretching active barnacle muscle fibers produced biphasic changes in free sarcoplasmic calcium: an increase followed by a decrease. This is consistent with the hypothesis that cross-bridge detachment decreases and cross-bridge reattachment and strain enhances calcium binding to the thin filament. A preliminary account of this work has appeared (Gordon and Ridgway, 1989).

**Methods**

The methods have previously been described in detail (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987). Single muscle fibers from the giant barnacle, *Balanus nubilus*, were
dissected, cannulated, and microinjected with the calcium-specific photoprotein aequorin (Shimomura et al., 1962). Purified aequorin C, chosen because of its high sensitivity and speed of response (Shimomura, 1986), was used for most studies, but the results were independent of the aequorin isoform used. Stretching or shortening current-clamped muscle fibers depolarizes or hyperpolarizes the membrane, affecting calcium release from the sarcoplasmic reticulum (SR) (Ridgway and Gordon, 1975; Gordon and Ridgway, 1976). Voltage clamping eliminates these length effects and was used in the experiments reported here. We did not measure sarcomere length, but adjusted fiber length to just detectable passive tension, which corresponds approximately to the in vivo rest length (sarcomere length ~7–8 μm; Griffiths et al., 1990). Muscle length was measured and controlled with an optical, electromechanical feedback circuit driving a shaker pot (V47/3; Ling Electronics, Royston, UK; see also Fig. 1 in Ridgway and Gordon, 1984). This allowed us to control and step the muscle length by up to 2 mm in <10 ms. Force was measured with a transducer (DSC-6; Kistler-Morse, Bellevue, WA). Light from the injected aequorin was measured by a photomultiplier having a 5-cm-diam photocathode. This was larger than the injected fiber length, so that the fraction of the total light emission it collected was independent of muscle length. The light signal was converted to a "calcium signal" by assuming that the aequorin light is related to free sarcoplasmic calcium as [calcium = K(light)] (see Blinks et al., 1982; Ridgway and Gordon, 1984). For these calculations, the total light intensity was used, including the resting glow, as in our previous studies (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987).

RESULTS

The Biphasic Response to Stretch

Fig. 1 compares the effects on the aequorin light signal of a 3% decrease in muscle length (A) and a 3% increase in muscle length (B) applied at the same time after the stimulus. For both records, the extra light in response to the length change has been computed by subtracting the control light record (no length change, shown in C) from that observed. Shortening (release) leads to an increase (extra light) as previously described (Ridgway and Gordon, 1984). Stretch produces a rapid increase (positive phase, as in shortening) followed by a negative phase of a transient decrease in intracellular calcium (negative phase). The few previous measurements of the response to stretch (e.g., see Fig. 3 of Ridgway and Gordon, 1984) were usually made at low amplitude, and at a time in the contraction when the positive phase was small (see Fig. 6 below). They show hints of the positive, but mainly a negative phase. Stephenson and Wendt (1984) also observed in skinned rat soleus muscle fibers loaded with the calcium-sensitive photoprotein obelin a small decrease in light upon stretch during calcium activation and an increase on shortening. Thus, the biphasic response to stretch is fundamentally different from the response to shortening.

Fig. 2 shows that stretch of a relaxed, unstimulated, voltage-clamped fiber (C, at the arrow) does not change the myoplasmic calcium. Also, no extra light is produced by stretching a relaxed fiber (B, at the arrow). The biphasic response (A) occurs only for stretches during the calcium transient; thus it occurs only in the active fiber.

The Response to Stretch Depends on Its Rate

Both phases of the extra light depend critically on the rate at which the fiber is stretched. When the rate of stretch is slowed the positive phase is decreased relatively
more than the negative phase (compare the rapid stretch in Fig. 3 B with the 200- and 400-ms duration stretches in Fig. 3, C and D). The positive phase occurs while the fiber is being stretched; the negative arises only after the stretch is complete. This is consistent with the hypothesis that the extra calcium is mobilized from thin filament sites because of the detached cross-bridges, the decrease in free calcium occurring as it binds to thin filament sites when cross-bridges reattach and are strained. Fig. 3, C and D, also illustrate the complex force response during a relatively slow stretch. The rate of force increase during the stretch abruptly decreases for stretches greater than ~0.2% muscle length (0.21 ± 0.06 [mean ± SD, n = 4]). This abrupt change in slope (or breakpoint) has been associated with
cross-bridge detachment in vertebrate skeletal muscle (Flitney and Hirst, 1978; Griffiths et al., 1980; Edman et al., 1978).

The Two Phases of Extra Light in Response to Stretch Have Different Dependences on Stretch Amplitude

Fig. 4 illustrates that the amplitudes of the positive and negative phases of extra light in response to rapid (<10 ms) stretches depend on the extent of stretch. Because the phases overlap, it is not possible to measure their individual amplitudes. Therefore, we have taken the maximum extra calcium as a measure of the positive phase amplitude and the minimum as a measure of the negative phase amplitude (see Fig. 5, left). The amplitudes of the positive and negative phases increase with stretch (Fig. 4). There appears to be a threshold stretch for the positive, but not for the negative
phase. This is seen in Fig. 4 where the length change (stretch) intercept for the positive phase is ~0.2% of muscle length, while it is near zero for the negative phase. The average stretch threshold for the positive phase for eight fibers is 0.41 ± 0.42% (mean ± SD) with all fibers showing a positive intercept. Six of the eight fibers have intercepts in the 0.12–0.31% range, but two fibers have intercepts of 1.0–1.16%, which makes the average higher and the variance larger. The overlap of the positive and negative phases may contribute to this large variance, but the intercept was positive for all fibers. There is some tendency for the intercept to be higher for larger forces, but this does not hold for all fibers. A positive phase in response to stretch occurring only with rapid stretches of more than ~0.2–1.0% of muscle length suggests that it is due to the decreased affinity of TnC for calcium when cross-bridges detach.

**The Positive Phase of Light on Stretch Is Similar to the Extra Light on Shortening**

Evaluating the two phases of the response to stretch is difficult because they overlap. However, the positive phases of the extra light resulting from the same amplitude of

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**Figure 3.** The response to stretch depends on the rate of stretch. This figure shows the extra light in response to stretches of the same amplitude, 2.1% of muscle length, at different rates: control (A), 80 mm/s (B), 2 mm/s (C), and 1 mm/s (D). The extra light in B–D was calculated by subtracting from the light record the light observed during the control stimulation with no length change (A). Note in C and D the breakpoint or the abrupt change in slope of the force during stretch. The light trace peak is clipped. Fiber resting glow, 0.2 V. Fiber length, 19 mm. Fiber weight, 43 mg. Calibration bar is 1.4 mm for length, 100 mV for voltage, 5.0 g for force, 0.2 V for light, and 400 ms for time. Temperature, 8°C.
stretch or shortening for length changes greater than ~2% have a comparable rising phase (Fig. 5, left). Subtracting the extra light on shortening from that on stretch results in an almost purely monophasic curve except at the beginning of the record (Fig. 5, right). Thus the biphasic curve of the extra light in response to stretch can be dissected into two monophasic curves: a positive one equal to the extra light on shortening peaking early, and a negative one peaking later. Because the rising phases of extra light on shortening and stretch are comparable and the number of cross-bridges detached for the same length change are also comparable, we assume that the extra light on shortening and stretching are equal for stretches >2%. Accordingly, the negative phase on stretch is given by the difference curve shown in Fig. 5 (right). Clearly, this assumption holds only for stretches far above the

\[ \text{Figure 4. The amplitude of both phases depends on the amplitude of stretch. This figure shows the peak increase (max) in intracellular calcium during the positive phase (+) and the maximum decrease (min) during the negative phase (o) plotted against the amplitude of length change, stretch, in percentage of muscle length for a typical fiber. The curves are extrapolated using a least-squares linear fit to the first three points to indicate the intercept on the length change, stretch amplitude, and axis. Note the small negative (0.08%) intercept for the negative phase and the positive intercept (0.19%) for the positive phase. The intercept for the negative phase for all fibers is both positive and negative and the average is not different from zero; for all positive phases it is always greater than zero, averaging 0.41% of the muscle length. Fiber length, 14 mm; weight, 31 mg. Temperature, 8–9°C.}\]
calcium rebinds after the stretch. Our hypothesis is that the rebinding of calcium (probably to troponin) is a result of enhanced calcium affinity of this thin filament structure caused by the reattached and strained cross-bridges or by the increased length.

The Time Courses of the Peak Amplitudes of the Two Phases Parallel Free and Bound Calcium, Respectively

If the positive phase of extra light on stretch represents calcium coming off TnC binding sites, its peak amplitude should be proportional to the bound calcium, and for stretches at different times these peak amplitudes should parallel calcium binding to thin filament TnC sites. (The integral of the extra calcium is arguably the more appropriate value, but since the curves have essentially the same shape, the peak is proportional to the area.) Likewise, if the decrease in light during the negative phase indicates enhanced filament binding, the time course of its peak amplitude should parallel the free myoplasmic calcium. The relative amplitudes of the phases vary for stretches at different times during the contraction (Fig. 6). Stretch during the rising phase of the calcium transient produces positive and negative phases with similar amplitudes. Stretch early in the decline of the calcium transient shows a greater

Figure 5. Comparison between the extra light observed on shortening and that observed during the positive phase on stretch. The left panel compares the extra light for shortening steps (dashed line) to the extra light for stretches of the same amplitude (solid line) for length changes amounting to 1.4, 2.7, 3.7, and 5.0% of muscle length. In the right panel of the figure are plotted the differences between the extra light on stretch and the extra light on shortening the same amplitude shown in the left panel. These differences (extra light on stretch minus that on release) appear to be almost purely monophasic. Fiber weight, 36 mg. Fiber length, 20 mm. Temperature, 8°C. Calibration bar 0.5 V for light, 400 ms for time.
positive than negative phase; stretches later in the calcium transient only cause a positive phase. This is quantitated (Fig. 5) by assuming that the positive phase is equal to the amplitude of the extra calcium on shortening the same distance for these large (>2%) length changes and calculating the negative phase by subtraction. The extra (or deficit) light is converted to [calcium] using the 0.4 power relation (see Methods). Fig. 7 shows the calculated peak amplitudes of both phases of the response to stretch as a function of the time of stretch for two fibers and compares them with the calcium transient (free calcium). The negative phase is more nearly coincident with the calcium transient (the free [calcium]), while the positive phase is delayed somewhat. This delayed time course is more like that of the formation of the CaTnC complex, which should be intermediate between free calcium and force (see Fig. 15 of Ridgway and Gordon, 1984, and Fig. 8 of Gordon and Ridgway, 1987). There may be also a small delay in the rise of the negative phase as well, paralleling the rise in force or strain in the cross-bridges. The data are consistent with the hypothesis that the positive phase is determined by the instantaneous bound calcium and the negative phase by the instantaneous free calcium.
The Extra Calcium during the Positive Phase of the Response to Stretch Depends on the Active Force in the Fiber

When the initial length of the barnacle muscle fiber is decreased immediately preceding stimulation, the peak force for a constant depolarization decreases. This gives rise to the length-force relationship. We reported that when the initial length was decreased, the extra calcium seen on a shortening step decreased in parallel with the peak force, consistent with both variables depending upon bound calcium (see Fig. 4 of Gordon and Ridgway, 1987, and Fig. 9 below). We investigated this effect.

![Figure 7](image_url)

**Figure 7.** The time course of the peak amplitude of the positive phase of extra light is delayed from the calcium transient while the peak amplitude of the negative phase is earlier and more nearly synchronized with the calcium transient. The peak amplitude of the positive (squares) and negative (triangles) phases are calculated from the biphasic response to a stretch of 2.9% (using the procedure illustrated in Fig. 5), plotted against the time of the stretch, and compared with the time course of the control calcium transient (free calcium) (solid line, scaled by a factor of 0.106 to have about the same amplitude for comparison purposes). The points for the extra calcium on release (squares, which are assumed to be equal to the peak positive phase on stretch; see Fig. 5) are fit with a least squares polynomial of order four plotted as the thin solid line. To compare the time course, the extra calcium on shortening (squares) was scaled (times 1.81) to have the same maximum amplitude as the peak negative phase on stretch (triangles). Note that the peak negative phase (triangles) rises and peaks earlier and falls earlier than the negative phase (squares). Its time course is more nearly like the time course of the free calcium transient (with a small delay on the rising phase), while the extra calcium on shortening (taken to be equal to the positive phase in response to stretch) (squares) has a much delayed time course more similar to that for bound calcium. Fiber resting glow, 0.2 V. Fiber weight, 44 mg. Fiber length, 20 mm. Temperature, 8–9°C.
for stretches. Fig. 8 A shows the response to a 2.0% stretch during the declining phase of the calcium transient. Fig. 8 B shows the response to the same stretch at the same time but from a 6% shorter initial length. The extra light is calculated by subtracting the control light record (same stimulation at the same initial length). Preshortening decreases both the extra light seen on stretch and the peak force. The control peak isometric forces (Fig. 9 B) and extra calcium in response to shortening steps or stretches are plotted for contractions at different initial lengths. The

Figure 8. Protocol used to measure the effect of the initial fiber length on the changes in intracellular calcium produced by stretch. A, Response to a 2.0% stretch initiated from the control initial muscle length. B, Response to the same 2.0% stretch initiated at the same time but starting from an initial length 6.0% shorter due to preshortening. C, Control record for A, the response to stimulation at the same initial length as in A but with no stretch. D, Control record for B; response to stimulation at the same 6.0% shorter initial length as in B but with no stretch. The extra light in A and B has been calculated by subtracting from the light record a control light record (from C or D) taken at the appropriate initial length. Calibration bar is 1.4 mm for length, 5 g for force, 1 V for light, 20 mV for voltage, and 400 ms for time. Fiber resting glow, 0.28 V. Fiber length, 20 mm. Fiber weight, 36 mg. Temperature, 8°C.

amplitudes were taken as the maximum and minimum of the biphasic response to stretch; no attempt was made to separate them as was done in Fig. 5, since the length changes and the maximum forces for the shortening steps and stretches were not equal. The extra light was converted to extra calcium. The peak isometric forces (Fig. 9 B) and the extra calcium (Fig. 9 A) for both shortening and stretch (positive and negative phase) decrease with the fiber length. When the extra calcium is normalized by dividing it by the peak force for each initial length, it is less dependent on initial
FIGURE 9. Both the positive and negative phases of extra light on stretch increase with increasing initial length in parallel with the increased active force. A, The amplitude of the extra calcium during the positive phase (max stretch extra calcium) (+) and the negative phase (min stretch extra calcium) (circles) in response to stretch (taken from the same record) and the extra calcium on shortening (max release extra calcium) (triangles) (taken from earlier records on the same fiber but with an increased stimulus amplitude) plotted against the initial muscle length. Data were taken from the same fiber and records shown in Fig. 8. B, The peak active isometric force (force) plotted against the initial muscle length for this fiber (at the same stimulus intensity used for the experiments shown on the left). Note that this stimulus intensity was higher for the control records taken for the shortening steps (triangles) than for the stretches (circles). C, The peak extra calcium (or deficit) normalized to the peak isometric force at that initial length for that fiber (peak extra calcium/force) plotted against the initial muscle length for peak extra calcium on stretch (max stretch/force) (+), decrease in calcium on stretch (min stretch/force) (circles), or extra calcium on shortening (max release/force) (triangles). Curves are fit by eye. Fiber length, 20 mm. Fiber weight, 36 mg. Temperature, 8°C.
length (Fig. 9 C; cf. 9, A and B). The extra calcium on stretch also correlates with the extra force seen immediately after stretch as well as the peak isometric force at the initial length (Fig. 9 B). These observations are consistent with force, extra calcium on shortening, and extra calcium on stretch all being related to one another possibly because all depend on the calcium bound to the activating sites.

The negative phase, like the positive, is affected by the initial length (Fig. 9 A). When normalized by dividing by the peak isometric force for that initial length, the normalized value is more independent of the initial length (Fig. 9 C), as was also true for the positive phase.

Another way to change the active force is to let the fiber shorten, then wait for force to redevelop. A large shortening step markedly reduces both the force and the extra light (positive phase) in response to stretch. The force then redevelops (Fig. 10 F) and the extra light (positive phase), in response to a subsequent stretch, increases in parallel with force redevelopment (Fig. 10 F). The response to a stretch alone is shown in Fig. 10 A. In Fig. 10 B it is seen that the fiber goes slack and force subsequently redevelops in response to a large shortening step (~12%). There is substantial extra light in response to this large shortening step (release). When this shortening step is followed by a smaller stretch (4%) at various times during force recovery (Fig. 10, C–E), the net extra Ca in the positive phase increases along with the redeveloped force (Fig. 10 F). The net extra calcium in response to stretch was calculated by subtracting the response to the shortening step alone (Fig. 10 B) from each of the responses to the shortening step and the restretch made at different times during the force redevelopment (Fig. 10, C–E). When the ratio of the net extra calcium in response to stretch to the extra calcium from stretch alone (applied at the same time) is plotted against the time of the stretch, the peak amplitude of the positive phase of the extra calcium recovers in parallel with the redeveloped force (Fig. 10 F). This is consistent with both the redeveloped force and the extra calcium from stretch depending on the same factor, possibly the bound calcium, and is similar to results when the second step is a shortening step (Gordon and Ridgway, 1987, Figs. 12–14).

The effect of the shortening step on the negative phase of the response to stretch is more difficult to assess because it depends on the previous positive phase. It is also decreased by the previous shortening step and recovers, but the effect cannot be readily quantified from these data.

The effect of previous shortening on the positive phase during stretches suggests that the extra calcium from shortening steps and stretches has a common source. We hypothesize that it is calcium bound to the activating sites on TnC. Another test of the hypothesis of a common source for the extra calcium is to reverse the order of the steps: stretch and then shorten (Fig. 11). Fig. 11 A is the response to stretch alone, and Fig. 11, B–D, are the responses to stretch followed at progressively later times by shortening (release) to the initial length. The net extra light in response to the release is obtained by subtracting the light record in response to the stretch alone (Fig. 11 A) from each of those in Fig. 11, B–D in response to the stretch and shortening. Thus the net extra light on release trace shows the net response to the shortening step alone without the response to stretch. As with the response to a stretch after a shortening step (Fig. 10), reduction of the next extra light on
FIGURE 10.
shortening by a previous stretch depends on the interval between the release and stretch. Fig. 12 illustrates this, showing the time courses of the control calcium transient, the extra calcium for the shortening step, and the net extra calcium for shortening steps when preceded by the stretch. Note that the net extra calcium on shortening is decreased when preceded by the stretch. Fig. 12 also shows the rate at which the fiber recovers the ability to elevate calcium in response to a shortening step. The half-time for recovery is ~50 ms. Similar results were obtained with this protocol using another fiber.

DISCUSSION

This paper describes changes in free myoplasmic calcium caused by an increase in muscle length during contraction. On stretch, additional free calcium appears, followed by a decrease to below the control level. This suggests that calcium is released, then rebound to the same or other sites in the muscle. Neither the SR nor the extracellular fluid are likely to be either the source or sink for this calcium, as argued previously for the extra calcium on shortening (Ridgway and Gordon, 1984). The myofibrillar calcium activating sites on the thin filament are the most likely candidates. The evidence for this includes:

1. The biphasic response occurs only in active fibers (Fig. 2).

Figure 10. (opposite) A shortening step markedly reduces both the positive and negative phases of the extra calcium in response to stretch. A, Response of a muscle fiber to a 4% stretch alone with no initial shortening. B, Response of a fiber to a 12% shortening step alone with no subsequent stretch. C–E show the response of a muscle fiber when the restretch is preceded 80 ms (C), 200 ms (D), and 400 ms (E) by a 12% shortening step (release) large enough to slacken the fiber. The extra light with release or stretch alone in A and B are calculated by subtracting from the light record a control light record with no length change. The extra light net on stretch records for C–E were obtained by subtracting the light response to the shortening step alone in B from the light response to both shortening and delayed stretch. This procedure gives the net extra light in response to the stretch alone when preceded by the shortening step. For comparison, the extra light with stretch alone for each of these three records (C–E) is plotted for a stretch at the same time as the restretch in C–E. In C this record is identical to the extra light with stretch alone in A. For D and E comparable stretches were done at the appropriate time, and the extra light with stretch alone is shown. By comparing the extra light net on stretch with the extra light stretch alone one can see how the preceding shortening step has influenced the response to the restretch and how much the response has recovered during the indicated delay. This is done more quantitatively from the extra light records by computing the extra calcium using the equation given in Methods. F, The time course of recovery of the extra calcium in response to stretch parallels the redevelopment of force after a large shortening step. The force trace from B in response to the shortening step is replotted. Superimposed on the force trace is the measured peak extra calcium (circles) during the positive phase of the net response to stretch shown in C–E normalized to the value it would have in response to stretch alone (also shown in C–E). This normalized value is plotted at the time of the stretch and scaled so that the maximum and minimum correspond to the maximum and minimum of force redevelopment. Fiber length, 19 mm. Fiber weight, 36 mg. Calibration bar, 50 mV for voltage, 1.4 mm for length, 5.0 g for force, 0.5 V for light, and 400 ms for time. Fiber resting glow, 0.2 V. Temperature, 8°C.
2. It occurs under voltage clamp and long after the membrane (and presumably the SR) has been stabilized by repolarization.

3. The amplitudes of both phases depend on muscle force (Figs. 8 and 10 F), but for stretches during the twitch the time course of the peak amplitudes of the two phases does not parallel force. The time course of the amplitude of the negative phase parallels free sarcoplasmic calcium and that of the positive phase is intermediate between free calcium and force (Fig. 7).

4. The positive phase on stretch for large stretches is similar to the extra calcium on shortening in the active fiber (Figs. 1 and 4), which results from calcium

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**FIGURE 11.** Protocol for measuring the interaction between stretches and shortening steps in affecting the extra calcium seen accompanying the length change. In A a 1.8% stretch is applied, and in B–D a subsequent reshortening (release) back to the control length is applied during the declining phase of the calcium transient with the time between the stretch and reshortening changed from 200 ms (B), to 100 ms (C), to 25 ms (D). All initial stretches occurred at the same time. The net extra light on release in all cases was calculated by subtracting from the given light record with the stretch and reshortening the light record in A with the stretch alone. This procedure allows us to measure the extra light seen with the shortening alone when preceded by the stretch so that we can compare it with what it would be without the preceding stretch. This net extra light on release has nearly the same amplitude in all panels despite the fact that the expected extra light with shortening alone would increase dramatically from B to D as the shortening step occurs earlier. Fiber length, 22 mm; fiber weight, 36 mg. Calibration bar is 2.8 mm for length, 10 g for force, 1 V for light, 100 mV for voltage, and 800 ms for time. Fiber resting glow, 0.28 V. Temperature, 8°C.
dissociating from the activating sites due to the cross-bridge dependent calcium binding (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987).

5. For multiple steps, the extra calcium on shortening and stretch interact (Figs. 10–12) as though both are supplied by the same pool of calcium.

![Graph showing the interaction between extra calcium on shortening and stretch](image)

**Figure 12.** A previous stretch decreases the extra calcium seen with a subsequent shortening step. This figure shows the interaction between the net extra light seen on stretches and shortening steps using the protocol shown in Fig. 11. The net extra calcium on shortening is calculated from the net extra light on release records shown in Fig. 11. In these records the extra light is calculated by subtracting the light trace in response to the stretch alone from the light record in response to the stretch and delayed reshortening. This procedure gives a net extra light signal for that shortening step (preceded by the stretch). This net extra light on shortening is always less than if there had been no prestretch. This net extra light produced by the shortening step (when preceded by a prestretch) is converted to extra calcium and is plotted (triangles) against the time of shortening for each delay between prestretch and shortening. The time of the prestretch is fixed (arrow). Also plotted is the extra calcium on shortening alone (squares, without the prestretch) observed for length changes at several different times during the calcium transient. To show the relative time scale of these events, the control (at constant length) calcium transient is plotted by calculating calcium from the aequorin light signal and expressing it as an increase above the resting level, scaled by 0.0825 to have about the same amplitude as the maximum extra calcium. Note that for shortening steps close to the stretch, the extra calcium is substantially below that without the stretch. The fiber recovers from this stretch with a half-time of ~50 ms. The fiber is the same one shown in Fig. 11. The data points have been connected by straight lines to emphasize the relative time courses.

These results strongly suggest that the extra calcium on stretch during the positive phase comes from the activating sites, and the decrease in free calcium during the negative phase is due to calcium rebinding to the same sites. These sites are probably on the TnC component of the thin filament protein troponin, since these barnacle
muscles are thin filament regulated (Dubyak, 1985) and contain troponin (Potter et al., 1986). In addition, selective extraction of TnC from barnacle skinned muscle fiber preparations removes the calcium sensitivity, which can be restored by re-adding TnC isolated from these muscles (Ashley et al., 1987).

Griffiths et al. (1980) suggested that when an active frog fiber is stretched by >2% cross-bridges detach, then rapidly reattach at a new site and remain strained. In frog muscle these reattached cross-bridges produce more force per bridge and are probably strained, because after the stretch muscle force, but not stiffness, is elevated (Sugi and Tsuchiya, 1988); in fact, stiffness is decreased along with filament overlap. This lack of change in stiffness needs to be verified for barnacle muscle. Our data suggest that stretch initially causes calcium dissociation from thin filament binding sites, followed by increased calcium binding to the same or other sites. This is consistent with the hypothesis that calcium binding to the activating sites decreases as cross-bridges detach and increases as cross-bridges reattach at a new actin site and are strained. This hypothesis explains the positive phase of extra calcium during stretch as being due to detaching cross-bridges that decrease calcium binding (elevating free calcium) and the negative phase as being due to reattachment of cross-bridges with the strain produced by the continued stretch increasing calcium binding to the thin filament activating sites. The dependence of calcium binding on cross-bridge attachment, first hypothesized by Bremel and Weber in 1972, is consistent with their data and our results on the extra calcium due to shortening (Ridgway and Gordon, 1984; Gordon and Ridgway, 1978, 1987). The hypothesis of increased calcium binding brought on by strained cross-bridges is new.

For the positive phase, cross-bridges are probably detached by the large amplitude (up to 5% of muscle length) stretches that produce the breakpoint (abrupt change in slope) in force during stretch (Fig. 3, C and D). The dependence of the positive phase on the extent of the stretch (Fig. 4) suggests that detaching force-producing cross-bridges produces this extra calcium. Fig. 4 shows that muscle stretches of more than ~0.4% (mean 0.41%) above the length change that produces the breakpoint in force during stretch (Fig. 3) are required to produce a positive phase of extra calcium. In frog muscles the length change required to produce this breakpoint is ~1–2% (Flitney and Hirst, 1978; Griffiths et al., 1980; Edman et al., 1981). This difference is reasonable; barnacle muscles have a sarcomere length three to four times that of vertebrate striated muscle (Griffiths et al., 1990).

The similar amplitudes of the positive phase in response to stretch and to a shortening step for length changes >2% also is consistent with both being caused by cross-bridge detachment. The positive phase in response to stretch has a threshold; the extra calcium in response to shortening does not. This is consistent with the same interpretation, since there should be a threshold stretch for detachment, while the strain in cross-bridges would be relieved by shortening in a continuous manner.

The positive phase depends on the force as determined either by the initial muscle length (Fig. 9) or during force redevelopment after a shortening step (Fig. 10 F). This is further evidence of its dependence on detachment of cross-bridges and supports the hypothesis that cross-bridge detachment decreases, and cross-bridge attachment increases calcium binding to thin filament TnC as proposed by Bremel and Weber (1972).
The negative phase is the major feature distinguishing the response to stretch from that to shortening. In shortening, the response is monophasic. Calcium is released from some binding site and is then taken back up by the same or another site (e.g., SR the calcium pump or another calcium binding protein). The negative phase shows additional calcium uptake above control levels. This could occur by the TnC binding site affinity increasing above the control level, increasing the filament bound calcium, and decreasing the myoplasmic calcium levels. This occurs when cross-bridges are strained by stretch and the resulting force is even higher than the isometric value for the stretched length. Therefore, elevated strain probably increases calcium binding as diminished strain decreases it.

If strained, attached cross-bridges increase calcium binding, and detached cross-bridges decrease calcium binding, one might expect that upon stretch of the active fiber, the myoplasmic free calcium would first decrease rather than increase. In other words, why is there a positive phase when stretching strains cross-bridges before it breaks them? A likely explanation for our observations lies in the fact that the mechanical, cross-bridge events are at least an order of magnitude faster than the calcium dissociation events. In a typical experiment, the muscle is stretched by 4% in 10 ms. However, only 500 µs is required to reach the 0.2% length change necessary to detach cross-bridges. Thus the elevated strain only lasts 500 µs before detachment occurs. The calcium dissociation rates from troponin, however, are of the order of 10–100/s (100–10 ms). If these calcium dissociation rates are the rate-limiting steps in determining the free calcium for a change in force, then clearly these slow steps and any other intervening steps severely filter the observed changes in free calcium. In addition, the cross-bridge reattachment rates during stretch are also very high (of the order of 1,000 s⁻¹ in frogs [see Colomo et al., 1988, 1989], comparable to phase two rate constants; these numbers may be comparable in barnacles as the phase two rate constants in frogs and barnacles are similar [see Griffiths et al., 1990]). Thus detachment and reattachment occur at a rapid rate during stretch, much faster than calcium dissociation. Calcium dissociation might then respond to some average strain, which is also elevated (see Colomo et al., 1988). However, one also needs to know the shape of the curve relating strain (force) to the dissociation rate of the calcium–troponin complex ($k_{off}$), as this is generally assumed to be the site of cross-bridge attachment dependent change. The association rate is assumed to be fast and limited only by the calcium diffusion rate and rate of removal of water of hydration from calcium. The functional dependence of $k_{off}$ on strain (force) has not been measured, but the data of Guth and Potter (1987) lead one to estimate that it declines with increasing strain (force), with a positive second derivative approaching a limiting value at high forces. If the $k_{off}$ vs. strain curve has this form, comparable decreases and increases in strain will have an asymmetric effect on the $k_{off}$ and free calcium, because decreases in force (strain) will produce a larger absolute change in the dissociation rate than increases in force (because of the positive second derivative with respect to strain, concaved upward). Thus, because the cross-bridge attachment–detachment changes are faster than the free calcium changes produced by changes in $k_{off}$, a net increase in $k_{off}$ will be seen for symmetrical changes in strain. Hence, one expects a net increase in free calcium, the positive phase, during stretch, followed by a negative phase when there is prolonged attachment at high strain. This
explanation is, of course, highly dependent on relative muscle properties and curve shapes. In this light, it is not surprising that the preliminary data of Stephenson and Wendt (1984) on large stretches of rat skinned soleus muscle fibers shows only a small, rapid negative phase.

It has been long known that length affects calcium sensitivity (Endo, 1972). In fact, Stephenson and Wendt (1984) hypothesized that length-dependent calcium binding could account for effects of length changes on the bound calcium as well as on length sensitivity. However, in a previous article (Gordon and Ridgway, 1987) we have given evidence that cross-bridge attachment plays the major role in the length effects on calcium binding, and Allen and Kentish (1988) have arrived at a similar conclusion using a different preparation. For the following reasons our present results further implicate the cross-bridges and argue that force or cross-bridge strain is a major factor in the enhanced calcium binding with stretch. First, if cross-bridge force or strain is the major factor, the amplitude of the negative phase will depend on the rate, not just the extent of lengthening. Both phases are sensitive to rate of lengthening (Fig. 3). Second, the time course of the peak amplitude of the negative phase is not the same as that of the free calcium (Fig. 7) but is somewhat delayed as is the rise in force. This is more consistent with increased calcium binding depending on cross-bridge attachment and strain rather than on increased length alone. Third, if cross-bridge strain (force) is the major factor, the decrease in calcium will depend on the sum of the stresses on the thin filament and, thus, the number of attached cross-bridges. This is the case with changes in the initial length (Fig. 9) and for redeveloped force after a shortening step (Fig. 10). In the latter, the negative phase as well as the positive is affected by the previous shortening step. If the calcium binding were only length sensitive, the negative phase would be insensitive to developed or redeveloped force. These three observations argue strongly that the effect of increased length to enhance calcium binding is mediated through force or cross-bridge strain.

Cross-bridge-dependent calcium binding to the activating sites could play a major role in increasing the positive cooperativity of activation, especially at low calcium levels. It could also prolong activation by enhancing the effect of the small amount of remaining calcium as well as binding it more tightly (Ridgway and Gordon, 1984; Gordon and Ridgway, 1987). Increased calcium binding with cross-bridge attachment is a consequence of the probable role of attached cross-bridges in regulating actin–myosin interaction in muscle (see Taylor, 1979).

We conclude that cross-bridge strain affects the thin filament, increasing the calcium binding by TnC. This implies that cross-bridge strain can be transmitted through the thin filament to increase calcium binding and, presumably, activate the filament. Does this play a role in the enhanced force upon stretching muscle fibers? This could be important for submaximal calcium activation, where increased calcium binding would increase activation. However, even during tetanic stimulation, stretch enhances force above the isometric value with both a transient and sustained component (Edman et al., 1978). Increased stiffness is not associated with either phase (Sugi and Tsuchiya, 1988), so increased numbers of attached cross-bridges cannot account for this force enhancement. Sugi and co-workers (Amemiya et al., 1988) explain this by hypothesizing displacement of the thin filament away from the
position equidistant from three thick filaments to a position where greater force can be produced in the interaction. Other explanations are possible. Julian and Morgan (1979) suggest that nonuniformities of sarcomere length could give rise to this apparent steady-state enhancement because of the nonlinearity of the load–velocity curve for lengthening. In the experiments of Edman et al. (1981) and Sugi and Tsuchiya (1988) stretch appeared to be rather uniform. We did not investigate this question and do not know the extent of nonuniformities in our fibers. Another possible explanation is that stretch changes cross-bridge properties. Griffiths et al. (1980) and Colomo et al. (1988) report that cross-bridges broken by strain can reattach at highly accelerated rates. This might imply altered cross-bridge properties leading to a higher steady-state force. Another possibility for the enhanced force with stretch is that stretch could shift the equilibrium between cross-bridges that are attached (and producing stiffness) but not producing force and those that produce high force (see Bagni et al., 1988). This might be due to a change in the cross-bridges or in the thin filament such that the strained thin filament interacts with the cross-bridges to produce a higher force. The data in this paper imply that strain affects the thin filament structure, which leads to a change in calcium affinity of TnC.

We thank Dr. Dennis Willows and the staff at Friday Harbor Laboratories for their hospitality. We thank Dr. O. Shimomura for the gift of native aequorin and for the gift of several purified molecular species of aequorin. We greatly appreciate the assistance of Mark Sather in the careful analysis of the data, whether in computer program writing, data plotting, or data measurement. The suggestions made by Dr. S. Price and Dr. D. Martyn on earlier versions of the manuscript have been extremely helpful. We also appreciate the assistance of Martha Mathiason in the preparation of the manuscript and figures and with writing computer programs used in the data analysis.

This work was supported by National Institutes of Health grants NS-08384 and AM-35597 and by a Grant-in-Aid from the Virginia Affiliate of the American Heart Association.

Original version received 8 December 1989 and accepted version received 29 May 1990.

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