

Muscle Calcium Transient

Effect of Post-Stimulus Length Changes in Single Fibers

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ABSTRACT We examined the effects of post-stimulus length changes on voltage-clamped, aequorin-injected single muscle fibers from the barnacle *Balanus nubilus*. Extra light (extra calcium) is seen when the fiber is allowed to shorten (a small percentage) during the declining phase of the calcium transient. The opposite is observed when the fiber is stretched. Increasing the extent of shortening increases the amount of extra calcium, as does decreasing the temperature. The extra calcium probably comes from the myofilaments and not from the sarcoplasmic reticulum because (a) there is a strong correlation between the extra calcium and the level of activation; (b) there is a strong correlation between the extra calcium and the amount of force redeveloped after a length change; and (c) the time course of the appearance of the extra calcium is intermediate between that of the free calcium concentration and that of force. We suggest (a) that the calcium binding to the activating myofibrillar proteins is sensitive to muscle length or muscle force, and (b) that there is a pool of bound calcium (activating calcium) that waxes and wanes with a time course intermediate between the free calcium concentration and force.

INTRODUCTION

It is well established that calcium activates muscle contraction (Ebashi and Endo, 1968). Elevation of myoplasmic calcium leads to calcium binding to myofilaments or sarcoplasmic proteins, and then, through a series of steps, to actin and myosin interaction and muscle contraction. Changes in intracellular calcium (calcium transients) preceding contraction have been detected in a number of different muscles, including toad skeletal muscle (Jobsis and O'Connor, 1966), barnacle striated muscle (Ridgway and Ashley, 1967), frog skeletal muscle (Blinks et al., 1978; Baylor et al., 1982a), and rat cardiac muscle (Allen and Kurihara, 1982), using various calcium indicators, including aequorin, arsenazo III, and antipyrilazo. The consistent finding is that the free calcium concentration increases in muscle before the force rises and declines before the force falls. Using measure-

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ments of the rate constants for calcium association and dissociation from troponin (Johnson et al., 1979), Robertson et al. (1981) have proposed models predicting the time course of changes in bound calcium levels during activation, but no direct measurements have been made in intact muscle fibers.

On theoretical grounds (Taylor, 1979; Adelstein and Eisenberg, 1980; Shiner and Solaro, 1982), changes in cross-bridge interactions would be expected to affect calcium binding to the activating sites. Indirect evidence for this comes from the studies of Bremel and Weber (1972) and Fuchs (1977), which demonstrated that rigor bonds enhance calcium binding to the isolated myofilaments and myofibrils of rabbit skeletal muscle. Additional indirect evidence for an effect of cross-bridge interaction on calcium binding comes from hysteresis measurements on intact and skinned barnacle muscle fibers (Ridgway et al., 1983). It will be shown in this paper that an additional calcium signal is observed when muscle length is changed during activation; there is an increase when the muscle is shortened and a decrease when the muscle is lengthened. In this paper we explore the properties of this calcium signal and relate it to filament-bound calcium. Preliminary reports of this work have appeared (Gordon and Ridgway, 1978, 1979; Ridgway and Gordon, 1981; Ridgway et al., 1983).

METHODS

Materials

We used large single muscle fibers from the barnacle *Balanus nubilus*. The barnacles were dredged from a depth of 20 m of water in Lopez Pass, WA, and maintained in running seawater at the Friday Harbor Laboratories, WA. The fibers selected weighed 30–60 mg, and were 18–28 mm long and 1–2 mm in diameter.

Procedures

The fibers were dissected, cannulated, and then microinjected with 0.3–0.4 μ l of an aequorin solution (see below) according to the methods of Ashley and Ridgway (1970). The preparation was placed into the chamber shown in Fig. 1, which is similar to that described by Gordon and Ridgway (1976). Most of our experiments were conducted under voltage-clamp conditions. For this purpose a double-spiral electrode (Hodgkin et al., 1952) was inserted along the length of the fiber. The uninsulated portion of the current wire was close to 20 mm long and the uninsulated portion of the voltage wire was ~3 mm long. The presence of the double-spiral electrode inside the fibers sets limits on the amount of shortening that can occur without damage to the tendon end of the fiber by the electrode. A similar, though less severe, difficulty occurs with stretch because of the high force, which can be momentarily imposed on the ligature securing the fiber to the cannula (see Fig. 1).

Temperature

Most of the experiments were done at 7–9°C. This is the natural range of the seawater temperature at 20 m in the San Juan Islands area in Washington (Waldichuck, 1955). This is also an ideal temperature range for investigating the extra light induced by shortening inasmuch as the magnitude of this phenomenon diminishes with increasing temperature (see Fig. 9).

Myoplasmic Calcium Detection

Although several modified aequorins are now available (Shimomura and Shimomura, 1982), we have used native aequorin (Shimomura et al., 1962; Shimomura and Johnson, 1969, 1976) to detect changes in myoplasmic calcium. The preparation of the injection solution and the methods for detecting the calcium-mediated light output were similar to

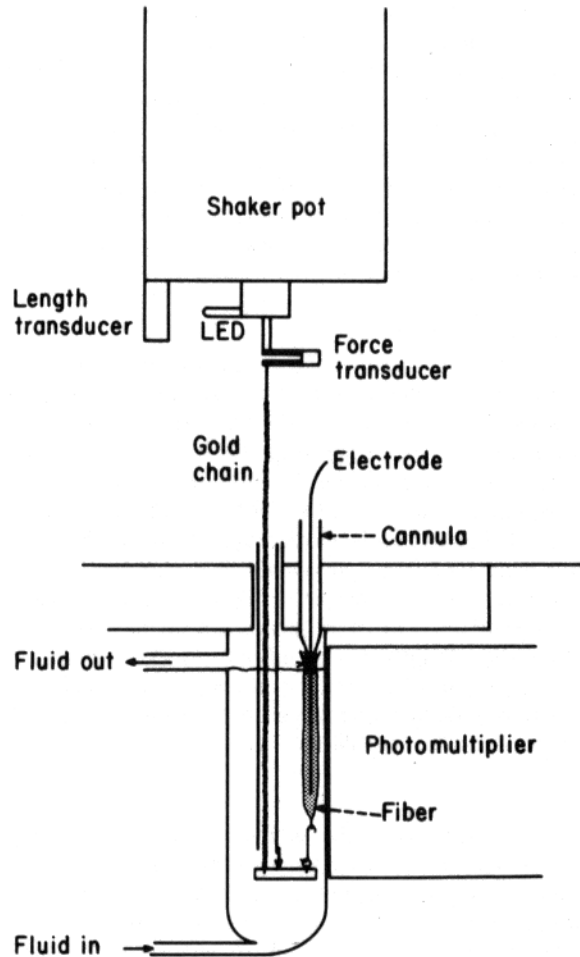


FIGURE 1. Diagram of experimental apparatus showing the cannulated fiber in front of the photomultiplier tube.

those described previously (Ashley and Ridgway, 1970; Gordon and Ridgway, 1976). To collect as much light as possible, the fiber was positioned close to (<2 mm from) the photomultiplier tube (PMT). Because our PMT has a 5-cm-diameter photocathode compared with a fiber length of ~ 2.5 cm, the entire fiber remains in view of the PMT despite changes in fiber length (see Fig. 1). Aequorin-injected barnacle muscle fibers give large, reproducible calcium transients with high signal-to-noise ratios (see, e.g., Fig. 2), so it is unnecessary to average.

Length and Force Measurements

Forces were detected with a DSC-6 transducer (Kistler-Morse, Bellevue, WA) with a linear output of 0–50 g force. Muscle length was measured with a PIN-SC-10 position transducer (United Detector Technology, Santa Monica, CA), which followed a light-emitting diode that was also mounted on the moving element of the electromechanical transducer (model V47/3 Shaker Pot; Ling Electronics, Royston, England; and see Fig. 1). Feedback from the length transducer was used to control the muscle length. The system was adjusted so that the response time for a test command step (a change of 1 mm in length) was always <10 ms, being typically 6 ms for a release and 8 ms for a stretch. Thus, our length changes are not fast when compared with, for example, those of Ford et al. (1977). They are rapid compared with the rate of tension development in these slow muscle fibers at 7–9°C (see Fig. 2, for example). To assess the response of the fibers to the imposed length changes, we examined a number of fibers (with a stereomicroscope at a magnification of 20) using carbon particles as landmarks. Our length changes were distributed over the entire length of the fiber (both at rest and during contraction) within the limits of our length excursions and the resolution of our microscope.

Data Collection

Membrane potential, membrane current, light (calcium transient), muscle length, and muscle force were recorded simultaneously. The data were digitized with a Tektronix Digital Processing Oscilloscope and the records were stored on a magnetic disk (4921; Tektronix, Inc., Beaverton, OR). Because our system has only four independent channels, all five measurements listed above could not be recorded for any one response. Although the channel isolation of our primary amplifiers (7A18; Tektronix, Inc.) is 50:1, the cross talk between the chopped signals can be noticeable. Accordingly, we have corrected all the records presented using a 1.5% proportional correction measured for our amplifiers. To eliminate any possibility for cross talk between length (or force) and light, they were recorded on separate amplifiers. The usual procedure for data collection is to obtain (a) a control record (at constant length, for example), (b) two experimental records (with length changes, for example), and (c) another control record. Thus, in any given series of records, fully one-third are controls, and for each experimental record, a control either immediately precedes or immediately follows.

Voltage Clamp

The voltage-clamp circuit used was similar to that described by Hagiwara et al. (1968). Currents were measured with a virtual ground system. The gain of the feedback circuit was adjusted so that membrane voltage could be controlled within $\sim 50 \mu\text{s}$. This effectively reduces the oscillations that are occasionally observed in some fibers (Gordon and Ridgway, 1978). In the present paper we present records from non-oscillating fibers only.

Voltage-clamp stimulation is needed because under conditions of constant current stimulation, the membrane potential of a barnacle muscle fiber is influenced by changes in length. As we have reported (Ridgway and Gordon, 1975; Gordon and Ridgway, 1976), the barnacle muscle fiber is extremely sensitive to pre-stimulus length changes that are made under constant current stimulation. For example, a 1% release in muscle length made just before the onset of stimulation can reduce the magnitude of the ensuing calcium transient and force responses by 40%. Pre-stimulus stretch has the opposite effect: it dramatically increases the calcium transient and force under constant current conditions. These length-sensitive properties of the barnacle fiber are due to a membrane component and are eliminated by voltage-clamping (Gordon and Ridgway, 1976). Voltage-clamping

allowed us to make pre-stimulus length changes in either direction without any effect on the ensuing calcium transient or force responses. Voltage-clamping was thus essential in this present study of the effects of length change on the filament-bound calcium.

Because of the clefts in these fibers (Hoyle et al., 1973) and the associated series resistance, there may be questions about the adequacy of voltage control and also about which membranes are being controlled (Keynes et al., 1973). The absence of oscillation in the voltage and the current records during the voltage clamp makes large instabilities unlikely. Additional experiments on the adequacy of voltage control will be described below. As regards the nature of the membrane whose voltage is controlled, the elimination of the length-sensitive calcium release under voltage-clamp conditions (Ridgway and Gordon, 1975; Gordon and Ridgway, 1976—referred to above) implies that these membranes are regulating excitation-contraction coupling. Presently available information does not allow us to distinguish among the various possible membranes (sarcoplasmic reticulum, sarcolemma, or clefts).

Calcium Gradients

Because of the nonlinearity of the aequorin reaction with calcium and possible calcium gradients in the muscle, one must be cautious when using the light signals to estimate the average, instantaneous Ca concentration (see Blinks et al., 1982, for a qualitative discussion of this point). The problems associated with calcium detection using aequorin to measure calcium movements across the surface membrane of the squid axon with long diffusion distances were discussed some years ago by Baker et al. (1971). In muscle the diffusion issue is somewhat different since there are multiple sites of Ca release and accumulation spaced at relatively short distances, the largest of which are of the order of half-sarcomere lengths and/or half-myofibrillar diameters, throughout the muscle. The morphology of the SR is organized, in part, to minimize the diffusion distances and to synchronize contractile activation. As a result, calcium gradients are usually expressed in terms of variations within a single sarcomere or single myofibril (Winegrad, 1970).

These calcium gradients are important for both contraction and relaxation, and we were naturally interested in the extent to which calcium gradients alone can explain the time course of our aequorin light signals or our extra light signals. To explore this issue we needed to calculate what the average diffusion distance for calcium would be from an internal release site during the time constant of the decline of the aequorin light signal. Einstein's (1905) equation, $X^2 = 2Dt$, relating the mean diffusion distance (X), to the diffusion coefficient (D), and the time (t), can be used as a first approximation. Assuming the bulk diffusion coefficient for calcium in sarcoplasm of 1.4×10^{-7} cm²/s (Kushmerick and Podolsky, 1969), and taking the time constant for the declining phase of the aequorin light signal as 250 ms, leads to a diffusion distance (X) of 2.7 μ m. This calculated diffusion distance is not inconceivably large, particularly along the longitudinal axis of these long sarcomered barnacle muscle fibers (Hoyle et al., 1973).

However, the above calculation of the length of the diffusion distance necessary to "explain" our light signals is probably underestimated for two reasons. First, the value assumed for the bulk diffusion coefficient was determined in fast frog fibers, where an active SR greatly reduces the observed diffusion distance and hence reduces the calculated diffusion coefficient. The active SR presumably restricts calcium diffusion between myofibrils, while allowing considerably freer diffusion within the core of the myofibril. Evidence from slower (tonic) frog fibers, which have less SR, shows calcium diffusing over much longer distances (Costantin et al., 1967), which implies a much higher diffusion coefficient in the presence of similar proteins but less SR. Measurements of diffusion coefficients in squid axon, where there is calcium binding and also active uptake (by

mitochondria), yields values from 10^{-6} (Hodgkin and Keynes, 1959) to 10^{-7} cm^2/s (Baker and Crawford, 1972) for diffusion over many hours. For diffusion over a shorter time period (as considered in this discussion), active transport would be minimized and diffusion coefficients should be somewhat higher (see Thomas, 1982). No direct measurements have been made in barnacle muscle under suitable conditions, but because we are considering times in the millisecond range, which are short compared with the hours for the diffusion studies mentioned above, the appropriate diffusion coefficient will be smaller than the value for calcium diffusion in water (5.3×10^{-6} cm^2/s [Robinson and Stokes, 1959]), but greater than the very low value found in fast frog fibers. We will assume it to be 1×10^{-6} cm^2/s for these calculations. Second, the time constant we determined from the fall in the aequorin light signal is an underestimation of the time constant for the fall in free calcium. This occurs because of the nonlinearity of the aequorin reaction (see below). The appropriate correction factor is ~ 2 , yielding time constants for the calcium signal of ~ 500 ms. We can therefore re-estimate the length of the diffusion path necessary to explain our aequorin light signals. With a diffusion coefficient of 1×10^{-6} cm^2/s and a time constant of 500 ms, the calculation gives a diffusion path of 10 μm . A path of this distance is unlikely.

As an alternative to the above calculation, we can ask how fast a calcium gradient of reasonable dimensions would be expected to disappear, once formed. Solving Einstein's equation for time ($t = X^2/2D$), assuming a distance of 1 μm , which is close to the radius of a myofibril, and taking the intermediate diffusion coefficient of 1×10^{-6} cm^2/s , gives a characteristic relaxation time of only 5 ms. Consequently, we believe that calcium gradients of reasonable size will self-destruct rather rapidly in barnacle muscle. Recalling that the response time of aequorin is limited to ~ 10 ms (see Blinks et al., 1978) and that the low-pass filter on our light channel has a cutoff of 10 ms, a 5-ms diffusion-controlled signal would be severely attenuated in our system. The effects of a 5-ms diffusion-controlled relaxation might not be severely attenuated in a faster system (e.g., Ford et al., 1977). These calculations suggest to us that the long-lived aequorin light signals and the extra light signals, which have time constants of ~ 250 ms in barnacle muscle, are not controlled by simple diffusion.

Further and stronger evidence against diffusion as the controlling (i.e., rate-limiting) process comes from experiments on the effect of temperature on the aequorin light signals. The rate constants for the declining phase of the aequorin light signal and the declining phase of the extra light signal are temperature sensitive. They have a Q_{10} of ~ 3.0 , which is similar to the 3.3 reported for SR calcium accumulation by Inesi and Watanabe (1967), but far different from the Q_{10} of 1.3 for the diffusion coefficient of calcium in free solution (Robinson and Stokes, 1959) and the Q_{10} of 1.5 for calcium binding to a typical myofibrillar protein, troponin C (Potter et al., 1977). Therefore, calcium accumulation by the SR, not diffusion, is probably rate-limiting during the declining phase of the calcium transient. The major calcium gradient at this time may well be across the SR membrane and not within the sarcoplasm.

On the basis of the above evidence, we doubt that changes in calcium diffusion gradients within the sarcomeres, amplified by the nonlinear response of aequorin, are controlling either the declining phase of the calcium transient or the extra light signals described in this article. Consequently, the relative changes in calcium concentration can be estimated from the extra light signals that accompany our length changes (see Fig. 15).

Saline

The physiological salt solution was prepared according to the formula of Hoyle and Smyth (1963), except that 4 mM TES [*N*-tris (hydroxymethyl) methyl-2-amino-ethanesulfonic acid], pH 7.5, replaced the bicarbonate.

RESULTS

Basic Effect of a Quick Release

Fig. 2 shows the effect of imposing a post-stimulus shortening step on a muscle at a time when the calcium transient is falling but while the muscle force is still increasing. Compared with the control record (at constant length, Fig. 2A), the

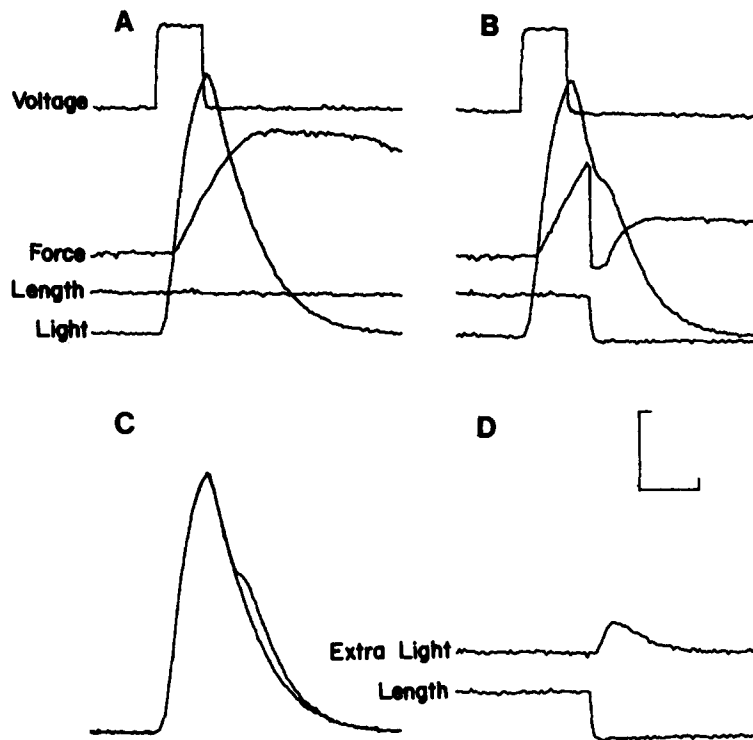


FIGURE 2. Effect of a quick release made during the falling phase of the calcium transient. (A) Control response; (B) response to a quick release; (C) superposition of the light traces (calcium transients) from A and B; (D) computer subtraction of the light traces from C to show the magnitude and time course of the extra light. Also included in D is the length step from B. Traces: membrane voltage at 40 mV/cal; aequorin light signal (calcium transient, unaveraged) at 1.0 μ A/cal; isometric force at 6.6 g/cal; fiber length at 2.5 mm/cal; extra light computer subtraction to obtain the extra light at 1.0 μ A/cal. Horizontal sweep rate, 400 ms/cal; temperature, 9°C; fiber length, 23 mm; fiber weight, 49 mg; resting glow, 200 nA.

length change (Fig. 2B) causes a burst of "extra" light, which appears as a hump on the falling phase of the calcium transient. This extra light can be more easily distinguished in Fig. 2C, where the calcium transient records from Fig. 2, A and B, are superimposed. We routinely compared the control and experimental calcium transients by subtraction. The resulting difference is shown in Fig. 2D ("extra light"), along with the step length change. The two main characteristics

of the extra light are (a) rapid onset after the length change and (b) an OFF time constant which is comparable to, but shorter than, the normal OFF time constant of the accompanying calcium transient. Overall, the time course of the extra light is much slower than the length change (see Fig. 2D). Finally, the force record shows an abrupt fall accompanying the shortening step, followed by force redevelopment to a value substantially below (B) the isometric value at the initial length (A) or the isometric value at the shortened length (not illustrated).

The immediate effect of a stretch (Fig. 3B) is a rapid increase of the force over the isometric control (Fig. 3A). Superimposition of the calcium transients

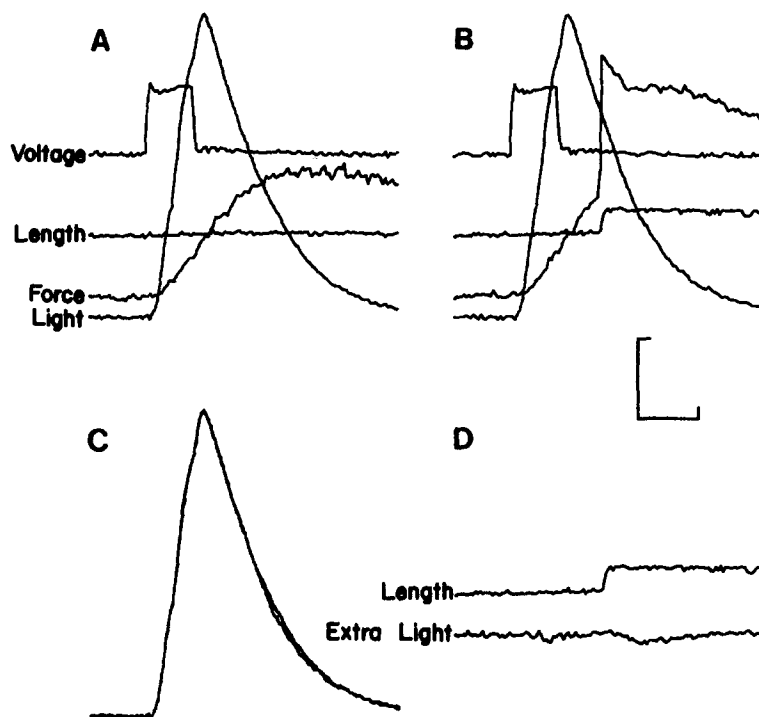


FIGURE 3. Effects of a quick stretch made during the falling phase of the calcium transient. (A) Control response; (B) response to a quick stretch; (C) superposition of the light traces (calcium transients) from A and B; (D) computer subtraction of the light traces from C to show the magnitude and time course of the extra light. Also included in D is the length step from B. Traces: membrane voltage at 40 mV/cal; aequorin light signal (calcium transient) at 0.4 μ A/cal; isometric force at 4.5 g/cal; fiber length at 2.1 mm/cal; extra light computer subtraction to obtain the extra light at 0.4 μ A/cal. Horizontal sweep rate, 400 ms/cal; temperature, 7°C; fiber length, 25 mm; fiber weight, 48 mg; resting glow, \sim 10 nA.

(C) and subtraction (D) show that the stretch causes a decrease in light. Thus, release and stretch have qualitatively opposite effects on the falling phase of the calcium transient (see Fig. 5 below). The effects of stretches, however, are less consistent and more likely to cause damage than releases. Accordingly, we have done most of our experiments with releases.

The Amount of Extra Light Is Related to the Extent of Release

Fig. 4 shows the effects of increasing the extent of muscle shortening. There is a progressive drop in force with greater extents of release (Fig. 4, B–D) until eventually the muscle is briefly slack (D). Also, the peak (absolute) force redeveloped decreases with successive increases in the extent of release. The greater the extent of release, the greater is the peak value of the extra light (B–D). The

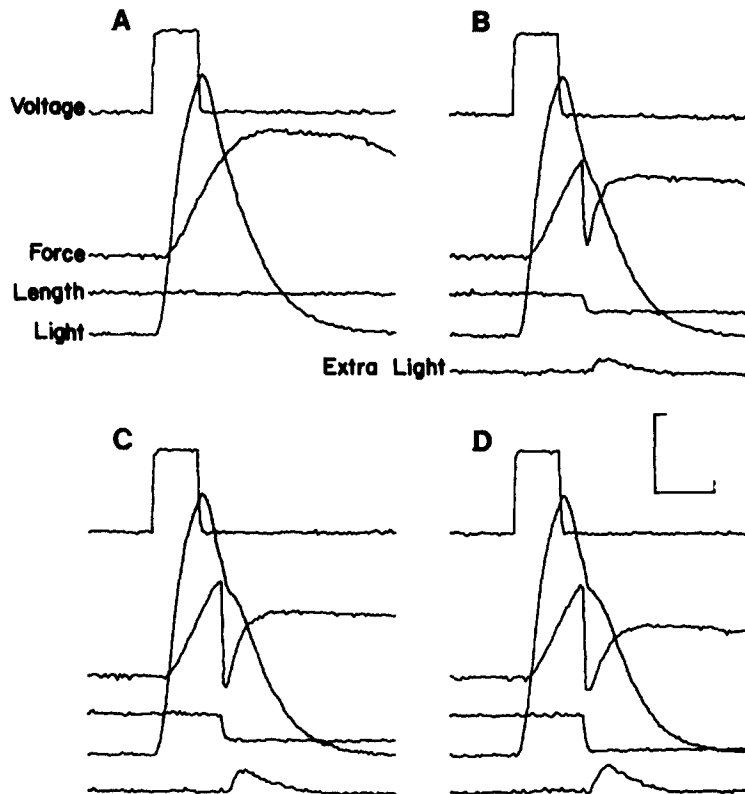


FIGURE 4. Effect of increasing the extent of the release. (A) Control response; (B–D) successively greater releases. Traces: membrane voltage at 40 mV/cal; light (calcium transient) at 1.0 μ A/cal; isometric force at 6.6 g/cal; fiber length at 2.5 mm/cal; extra light by subtraction at 1.0 μ A/cal. Horizontal sweep rate, 400 ms/cal; temperature, 9°C; fiber length, 23 mm; fiber weight, 49 mg; resting glow, 200 nA.

largest release (D) yields an extra light signal whose time to peak is slightly longer. This might be due to the muscle having gone slack and therefore having shortened for a longer time, with additional light production during this extended period of shortening (see Discussion). The qualitative symmetry between stretch and release is illustrated in Fig. 5. The extra light seen with a given release is greater than the decrease in light production associated with the same amount of stretch. We are not able to demonstrate saturation of the extra light for either stretch or release, although the curves do flatten out substantially at

either extreme. This is partly due to the limitations of the preparation and chamber, which prevented us from imposing length changes greater than ~2 mm (~10%; see Methods). As will be argued below in the Discussion, force may influence calcium binding, and, as long as cross-bridge cycling is maintained, force, and presumably calcium binding, will be low.

The extra light depends on the rate of release if the release occurs slowly compared with the maximum velocity of active shortening of the muscle. In four experiments, fibers were allowed to shorten a distance of 0.5 mm at different

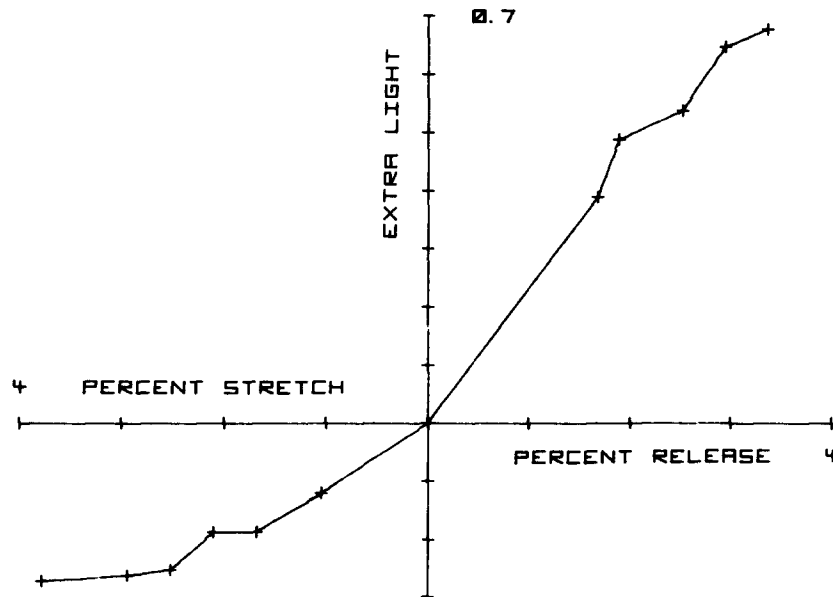


FIGURE 5. Effects of stretch and release are compared. The ordinate is the amplitude of the extra light signal (negative for stretch) in arbitrary units. The light at the time of the length change is 5.2 in the same units. The abscissa is the percentage change in muscle length, made in an experiment similar to that shown in Fig. 4.

rates during the declining phase of the calcium transient. Virtually the same extra light is seen for releases having durations between 10 and 100 ms, corresponding to shortening rates of >0.25 muscle lengths/s. Slower releases produce correspondingly less extra light. Crude estimates of the maximum shortening velocity in these experiments over the temperature range of 3–9°C using the Edman (1979) technique gave values between 0.12 and 0.3 muscle lengths/s, which is approximately the shortening rate above which the extra light is insensitive to the shortening rate.

Pre-Stimulus and Passive Length Changes

We have previously shown (Gordon and Ridgway, 1976) that pre-stimulus release and pre-stimulus stretch do not substantially alter the peak value of the ensuing

calcium transient, although they do have an effect on the peak (absolute) value achieved. Fig. 6, *A* and *B*, demonstrates this and shows that there is no effect of initial length on either the peak value of the light or on its time course. In addition, the length changes have no detectable effect on the light emission

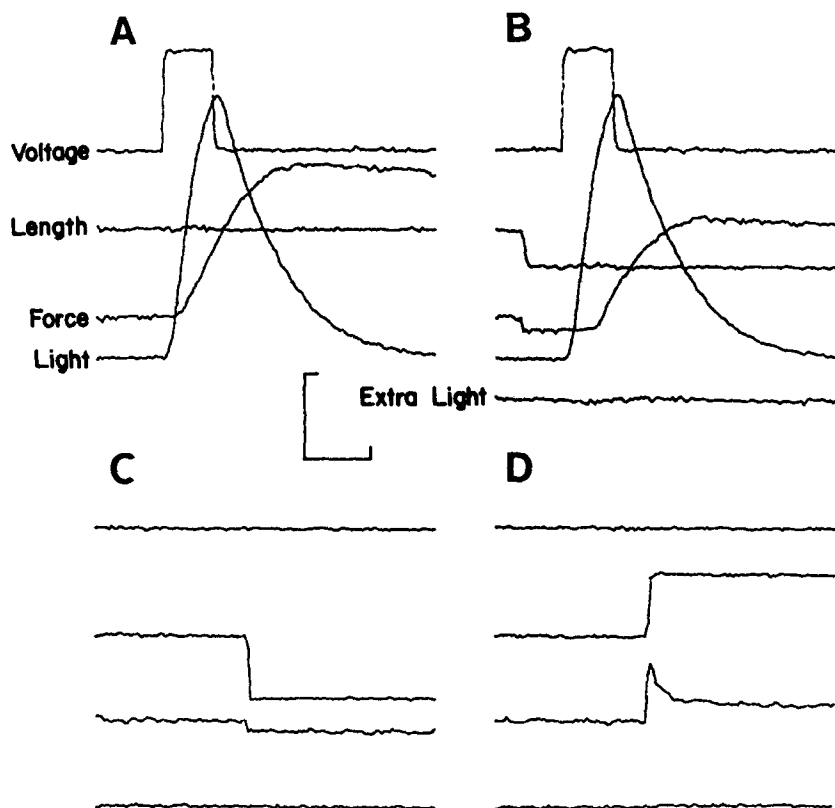


FIGURE 6. Control experiments. (*A*) Control; (*B*) pre-stimulus release. Traces: membrane voltage at 40 mV/cal; aequorin light at 1.0 μ A/cal; isometric force at 6.6 g/cal; muscle length at 2.5 mm/cal; extra light at 1.0 μ A/cal. Horizontal sweep rate, 400 ms/cal; temperature, 9°C; fiber length, 23 mm; fiber weight, 49 mg; resting glow, 130 nA. (*C*) Passive (unstimulated) release; (*D*) passive (unstimulated) stretch. Traces: membrane voltage at 40 mV/cal; aequorin light signal (calcium transient) at 1.0 μ A/cal; isometric force at 4.55 g/cal; muscle length at 1.05 mm/cal. Horizontal sweep rate, 400 ms/cal; temperature, 3°C; fiber length, 27 mm; fiber weight 60 mg; resting glow, \sim 100 nA.

(light) in unstimulated fibers. This can be seen in the early part of the records (*A* and *B*) and is confirmed for large passive releases and stretches (*C* and *D*). These records do show an effect of the length change on the force, but none on the light. In addition, these control length changes were made at 3°C, where the extra light accompanying the length change is greater than at higher temperatures (see Fig. 9 for temperature sensitivity).

Control Experiments

Because the extra light signals shown in Figs. 2 and 3 are small compared with those resulting from the ordinary calcium transients, we have done an extensive series of control experiments to demonstrate that the extra light is actually a property of the fibers. For clarity we divide the control experiments into two classes, mechanical and electrical.

MECHANICAL CONTROLS Our main concern here arises from the possible damage artifact caused by the presence of the double-spiral axial electrode. Obviously, from Fig. 1, when we change length, the fiber must slide over the axial electrode; therefore, the possibility of damage must be rigorously excluded. The damage hypothesis can only explain positive extra light, since damage of the SR or surface membrane would allow additional calcium to escape into the myoplasm. The stretch-related decrease in light (negative extra light, Fig. 3) cannot be explained by mechanical damage since this would imply a "negative damage." We have done the control experiment of removing the internal axial electrode altogether. The fiber must then be stimulated with external plate electrodes (Hoyle and Smyth, 1963). Under these conditions, the extra light signals are also observed (see Fig. 7). External plate electrodes have not been used throughout the present study because plate electrodes lead to inconsistent stimulation, which in turn gives inconsistent activation, which results in variable contraction (C. C. Ashley, E. B. Ridgway, and A. I. Selverston, unpublished results). Consistency of activation and contraction is very important in the present study since the extra light is revealed by subtraction of the control record (see Figs. 2 and 3). Passive stretch or release of the unstimulated fiber produced the expected changes in force, but no change in light (see Fig. 6, *C* and *D*). Thus, the mere movement of the fiber over the electrode cannot be the cause of the extra light observed during release of active tension.

We also considered the ligatures at the cannulated (shell) end and the tendon end of the fiber as possible sources of artifact. To control for these possibilities we masked off both the cannulated and the tendon end of the fiber from the PMT (see Fig. 1). With the masking in place, decreases in length during active tension still induced extra light, which demonstrates that the extra light is not coming from either end of the fiber. Because the PMT can normally collect light from the entire length of the fiber, we decided not to use the mask when collecting data because once the fiber ends are masked, the length change results in more or less of the fiber coming into "view" of the PMT. For simplicity, we have chosen to present data exclusively from unmasked fibers, with the entire fiber in view of the PMT before, during, and after the length change. It should be noted in this connection that the observed extra light signals are much larger on a percentage basis than are the changes in length that produce them. For example, Fig. 4*D* shows a 5% shortening step leading to a 15% change in the light signal.

Finally, we occasionally damaged a fiber, either inadvertently, e.g., with overstretch, or intentionally during the insertion of the axial electrode. Light signals from damaged fibers have three important characteristics: (*a*) they are generally large, (*b*) they have time constants for recovery of several hundreds of

seconds, and (c) they are highly variable. In contrast, the extra light signal seen on length change (e.g., Fig. 2) is small, has a time constant of the order of 200 ms, and is highly consistent.

ELECTRICAL CONTROLS As was previously indicated, voltage-clamping is necessary to achieve consistent stimulation and to eliminate the effects of initial length on the calcium transient. In addition, the voltage clamp serves several other purposes. Most of the length changes are made after the end of the stimulus

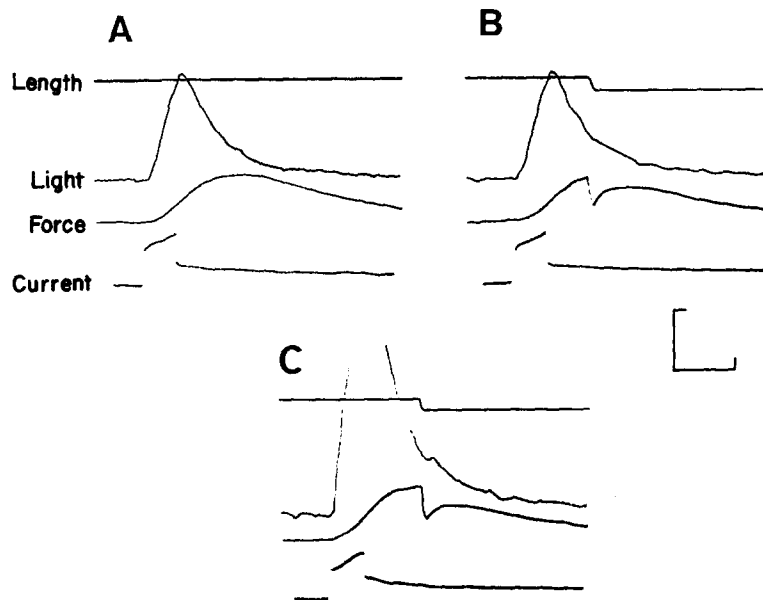


FIGURE 7. Records of the effects of length change in the absence of the internal electrode (stimulation via external plate electrodes). Traces: muscle length at 1.5 mm/cal; aequorin light at 400 units/cal; isometric force at 3.3 g/cal; total current at 10 arbitrary units/cal. In C, the light gain is doubled and the current gain is halved. Horizontal sweep rate, 400 ms/cal; temperature, 13°C; fiber weight, 45 mg; fiber length, 30 mm; resting glow, not recorded (1974, VII-13).

pulse (see Figs. 2 and 3). At that time, the voltage clamp is keeping the membrane potential at the holding potential, far (10–20 mV) from the threshold for calcium release and even farther from the threshold for contraction. This suggests that the effect of the length change is on the myofilaments and already renders the sarcoplasmic reticulum (SR) an unlikely source of the extra calcium. To further investigate the possible role of the SR in these post-stimulus length changes, post-stimulus hyperpolarizing pulses were used (see Fig. 8). When the membrane was immediately hyperpolarized after the stimulus pulse and kept hyperpolarized until the calcium transient was completed, no effect was observed on (a) the time constant of the falling phase of the calcium transient, (b) the time constant of the relaxation of force, or (c) the magnitude, duration, or time constant of the extra calcium seen on release. The inference is that the SR calcium release

mechanism is inhibited when the voltage clamp returns the membrane potential to resting levels.

Decreasing the Temperature Increases the Amplitude of the Extra Light

The amplitude of the extra light for a given length change is greater at low temperatures than at high. Fig. 9 shows results for 12 and 5°C, where the extra light is increased by a factor of ~2 for the 7°C temperature change. Both the

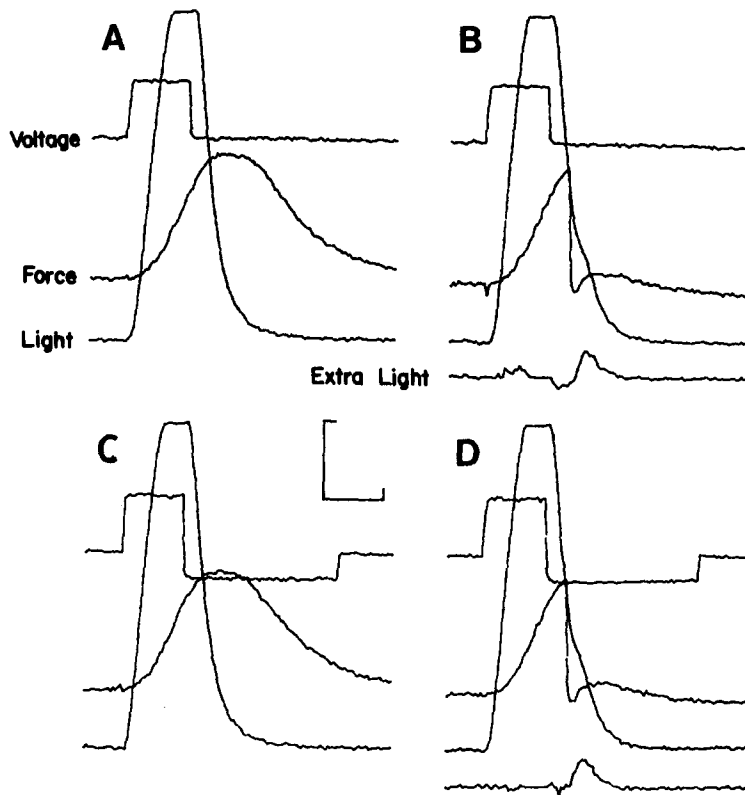


FIGURE 8. Constancy of the falling phase of the aequorin light and the extra light. (A) Control; (B) control with length change; (C) hyperpolarizing post-pulse; (D) hyperpolarizing post-pulse with length change. Traces: membrane voltage at 40 mV/cal; aequorin light at 0.2 μ A/cal; isometric force at 9.1 g/cal; extra light at 0.2 μ A/cal. Horizontal sweep rate, 400 ms/cal; temperature, 10°C; fiber length, 21 mm; fiber weight, 73 mg; resting glow, 52 nA. The peaks of the light traces have been clipped by our A/D converter.

calcium transient and contraction occur more slowly at the lower temperature. The time constants for the decline of the calcium transient and force have Q_{10} 's of ~3.0 and 2.3, respectively. In addition, because of the slower decline of the calcium transient at the lower temperature (presumably caused, in part, by slower calcium accumulation by the SR), the area of the extra light signal increases more than the amplitude. At unphysiologically high temperature, e.g., 20°C, the

extra light is scarcely detectable. For these reasons and others, most of our experiments were carried out at 7–9°C, the in situ temperature for barnacles in the San Juan Islands (Waldichuck, 1955). The observed increase in the amplitude of the extra light with decreasing temperature may be underestimated because the velocity of the aequorin reaction decreases with decreasing temperature (Allen and Blinks, 1979; Hastings et al., 1969).

The Amplitude of the Extra Light Depends on the Level of Calcium Activation of the Myofilaments

One way to increase calcium activation and force is to increase the stimulus intensity. Fig. 10 shows data taken at two stimulus intensities, which led to peak force responses in the controls of 7.5 and 16.5 g. The higher stimulus intensity

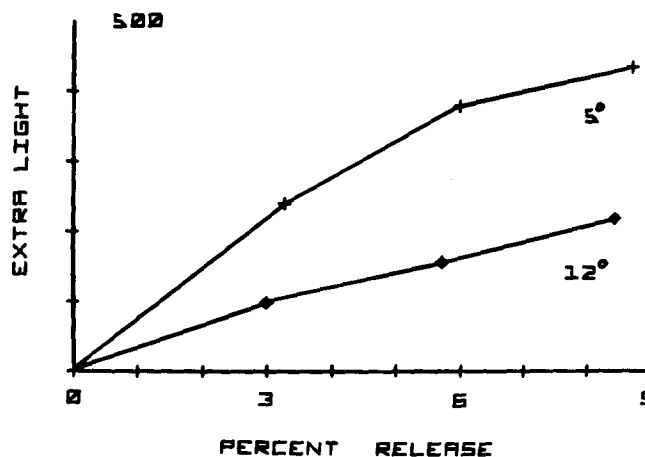


FIGURE 9. Relation of the amplitude of the extra light to the extent of release at two temperatures in the same fiber. The stimulus intensity was adjusted to give approximately the same amplitude light signal at the two temperatures, and the length changes were made at about the same light level during the declining phase of the calcium transient (at 5°C, 2,300 units and at 12°C, 2,000 in the same arbitrary units as the extra light). For these stimuli, the peak force amplitude was 20% greater at 5°C. Fiber length, 23 mm; fiber weight, 54 mg; resting glow at 5°C, 100 nA; resting glow at 12°C, 150 nA.

results in a larger amplitude calcium transient and more force, presumably because more calcium is bound to the myofilaments. A given percentage release (initiated at the same post-stimulus time) results in more extra light at the high than at the low level of activation.

A second way to increase muscle force is through repetitive (double-pulse) stimulation to produce a summation of tension. Fig. 11 shows data from such an experiment with and without length changes. It demonstrates several aspects of the double-pulse stimulation. The amplitude of the calcium transient elicited by the second stimulus pulse is smaller than that elicited by the first, even though it sits on the tail of the first. The excitation-contraction coupling mechanism, whatever it may be, needs time to “reprime” between stimuli (Hodgkin and

Horowicz, 1960). The calcium transients are close enough in time to sum slightly, but there is greater summation of force, so that substantial additional force is generated by the second calcium transient. If we allow a quick release (shortening) during the falling phase of the calcium transients, at times marked either *A* or *B* (initiated at equivalent amplitudes on either the first or second calcium transient), then more extra light is seen when the release is made at point *B*. Fig. 12 shows these data for the experiment shown in Fig. 11, and data from releases made at other times during both the first and second calcium transients. The important

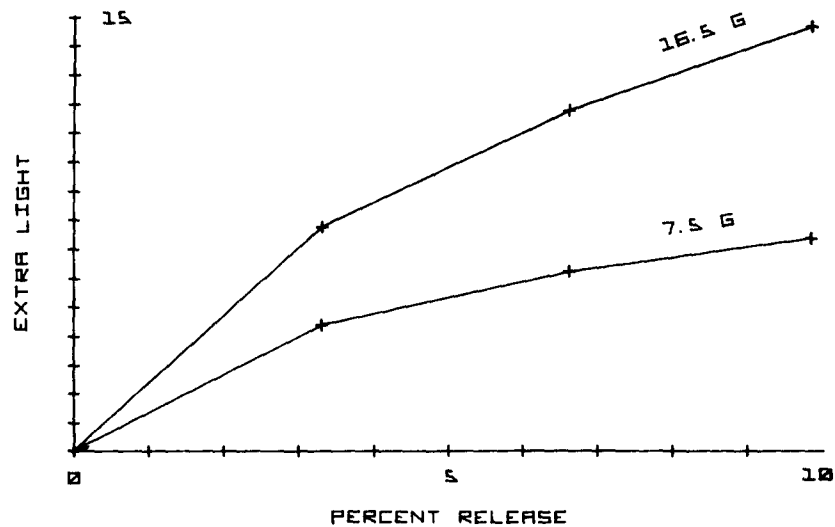


FIGURE 10. Effect of the level of calcium activation on the amplitude of the extra light seen on release. The forces assigned to the curves refer to the peak force achieved in the control contraction. For the two levels of stimulation, releases of differing amplitudes were performed at the same post-stimulus time. The light at the time of the release was 70 and 29 for the higher and lower forces expressed in the same units as the extra light. Fiber length, 20 mm; fiber weight, 55 mg; temperature, 9°C; resting glow, 180 nA.

point shown in these two figures (11 and 12) is that even when the ability of the SR to release calcium has been compromised, the amount of extra light seen on shortening is increased. This finding renders the SR an unlikely source of the extra light.

The Timing of the Length Change

When a fiber is released at various times from early in the contraction (when force is low but the calcium transient is high) until late in the contraction (when force is high but the calcium transient is low), the amount of extra light seen varies systematically. Fig. 13 shows a series of records wherein a given release is stepped through the contraction. When the release is delivered late in the contraction (Fig. 13*B*), the extra light is relatively small compared with that seen for a release delivered early in the contraction (Fig. 13, *D* or *E*). For very early

releases (Fig. 13*F*), the amplitude of the extra light declines somewhat. This is discussed below.

Correlation with the Amount of Force Redeveloped

The force record in Fig. 13 shows that after the length change the force falls to zero and then redevelops to a value which is below that in the control (Fig. 13*A*) under isometric conditions. For releases made early in the contraction (Fig. 13,

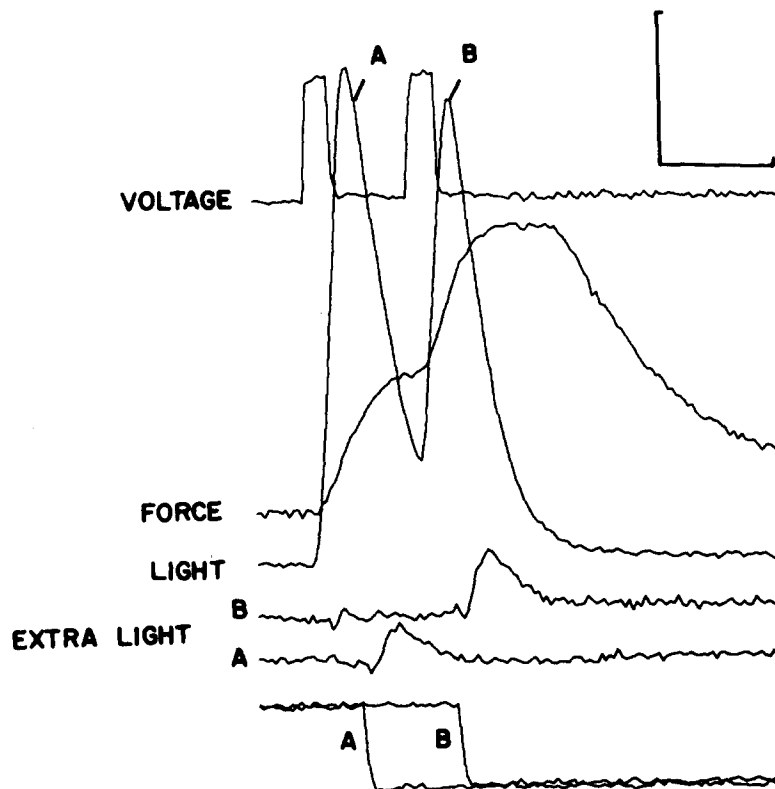


FIGURE 11. Paired stimuli showing force summation. Traces: membrane voltage at 40 mV/cal; light (calcium transient) at 0.4 μ A/cal. Points *A* and *B* show identical Ca levels on the first and second pulses at which length change is initiated. Isometric force at 3.8 g/cal; extra light subtraction of control light from light records with length change at *A* or *B*, 0.4 μ A/cal; fiber length for data records with length change at times *A* and *B*, 2.5 mm/cal. Horizontal sweep, 400 ms/cal; temperature, 9°C; fiber length, 23 mm; fiber weight, 54 mg; resting glow, 170 nA.

F and *E*), more force is redeveloped than when the release is made late in the contraction (Fig. 13, *C* and *B*). The amount of force redeveloped is expected to be closely correlated with the bound calcium (a more modern interpretation of the classical active state [Hill, 1969]). If the extra light (seen on release) samples calcium bound to the filaments, a correlation between the amount of redeveloped force and the amount of extra light seen on shortening is expected. Fig. 14

shows this correlation and also shows that this correlation is maintained over different intensities of stimulation and peak forces.

Bound Calcium Is Intermediate Between the Calcium Transient and Force

In experiments such as that shown in Fig. 4, it is possible to directly compare the amplitudes of the extra light signals because they are all obtained at the same instantaneous value of the calcium transient. However, in the case of Fig. 13, this procedure is invalid (even in a qualitative sense) because each of the extra

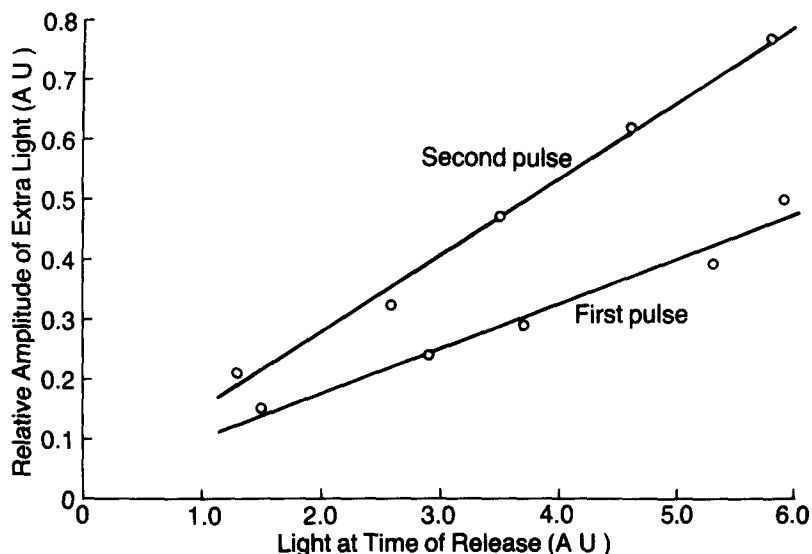


FIGURE 12. Extra light for paired stimuli: comparison of release on first and second pulses. The relative amplitude of the extra light is plotted against the amplitude of the calcium transient (light) at the time of the length change of 0.6 mm applied during the declining phase of the calcium transient after the first stimulating pulse (curve labeled first pulse) and after the second stimulating pulse (curve labeled second pulse). Points A and B in Fig. 11 would represent the same point on the abscissa. Same fiber as in Fig. 11.

light signals is obtained at a different instantaneous value of the calcium transient. A valid comparison can be made, in principle, by quantifying the extra light signal in terms of extra calcium. This quantification procedure requires detailed understanding of the reaction between aequorin and calcium, which, in its entirety, is undoubtedly complex. In the case of the extra light signals shown in Fig. 13, which are small perturbations occurring within the linear portion of the log luminescence vs. log [Ca] relationship,

$$[\text{Ca}] = k(\text{light})^n, \quad (1)$$

where [Ca] is the calcium concentration, light is the luminescence, which is the sum of two terms—the resting glow (Rg) and the light seen upon stimulation, the calcium transient (Ct)— k is a proportionality constant, and n is somewhere

between 0.5 and 0.4 (Baker et al., 1971; Allen et al., 1977). There is some uncertainty as to the exact power for a specific set of conditions and, in particular, it is important to take into account the magnesium concentration, ionic strength, pH, etc. (Ashley and Moiescu, 1977; Blinks et al., 1982), but reasonable estimates can be made for these values. We have chosen $n = 0.5$ for simplicity.

Over the range of calcium concentrations where Eq. 1 is applicable, small changes in calcium concentration are given by

$$\Delta[\text{Ca}] = k[(\Delta\text{Ct} + \text{Ct} + \text{Rg})^n - (\text{Ct} + \text{Rg})^n], \quad (2)$$

where ΔCt is the extra light which, together with the other parameters on the right-hand side of Eq. 2 (except k), can be measured from our records. Although

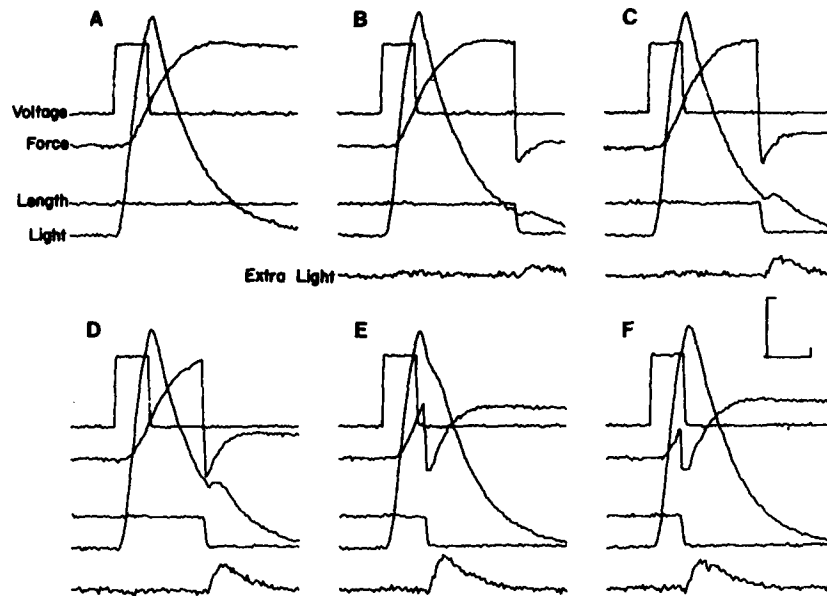


FIGURE 13. Effect of variation in the timing of the release. (A) Control; (B-F) releases at successively earlier times in the contraction. Traces: membrane voltage at 40 mV/cal; light (calcium transient) at 0.66 $\mu\text{A}/\text{cal}$; isometric force at 6.6 g/cal; fiber length at 2.5 mm/cal; extra light at 0.5 $\mu\text{A}/\text{cal}$. Temperature, 9°C; fiber length, 23 mm; fiber weight, 49 mg; resting glow, 120 μA .

k can be estimated from the resting light level, the exact value is not needed to compute relative values of calcium.

In Fig. 15A we show the time courses of the relative free calcium, the relative extra calcium, the control isometric force, and the length steps for releases at different times during contraction (as in Fig. 13). The relative free calcium and extra calcium have been calculated using Eqs. 1 and 2 with $n = 0.5$. In Fig. 15B we show the aequorin light signal and the extra light from which the relative free calcium and extra calcium are derived. The extra calcium is plotted as occurring at the time of the length step. The important point demonstrated in

Fig. 15A is that the extra calcium, presumed to reflect bound calcium, occurs as an intermediate between the calcium transient and the force. This conclusion is insensitive to the assumptions made regarding either the exact stoichiometry of the aequorin reaction with calcium or the exact value of the proportionality constant, k .

DISCUSSION

A major question concerning the extra calcium seen when the muscle is allowed to shorten during contraction is what its source is. We have presented evidence that it is not an artifact caused by damage, movement, or light from the ends of

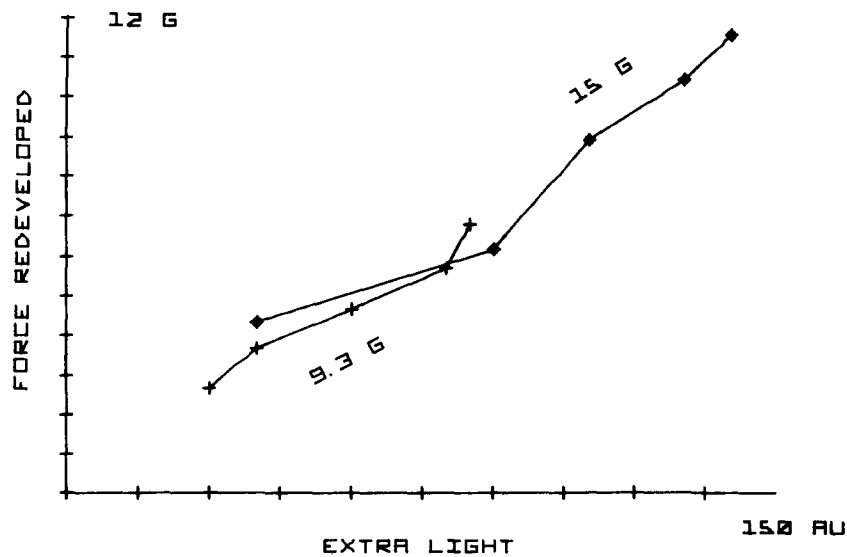


FIGURE 14. Correlation of redeveloped force with extra light. The abscissa shows the amplitude of the extra light seen after a shortening step of 0.8 mm imposed at various times during the declining phase of the calcium transient. The ordinate shows the peak redeveloped force after the shortening step. Two stimulation intensities were used producing a maximum force of 9.3 (crosses) or 15 g (squares). For each stimulus intensity, each point results from a shortening step at a different time. Fiber length, 24 mm; fiber weight, 55 mg; temperature, 8°C; resting glow, 70 nA.

the fiber, but is a real increase in calcium in the whole muscle fiber. From which of the calcium pools does it come?

The extra calcium probably comes from within the fiber and not across the surface membrane. This is because there is an increased outward current, accompanying the length change, when the muscle fibers are voltage-clamped (Gordon and Ridgway, 1976). Although we cannot definitively rule out a sodium:calcium exchange with a coupling ratio of >2 , it is rather difficult to see, for example, how such an exchange might depend on force, or how such an exchange might be induced by a length change.

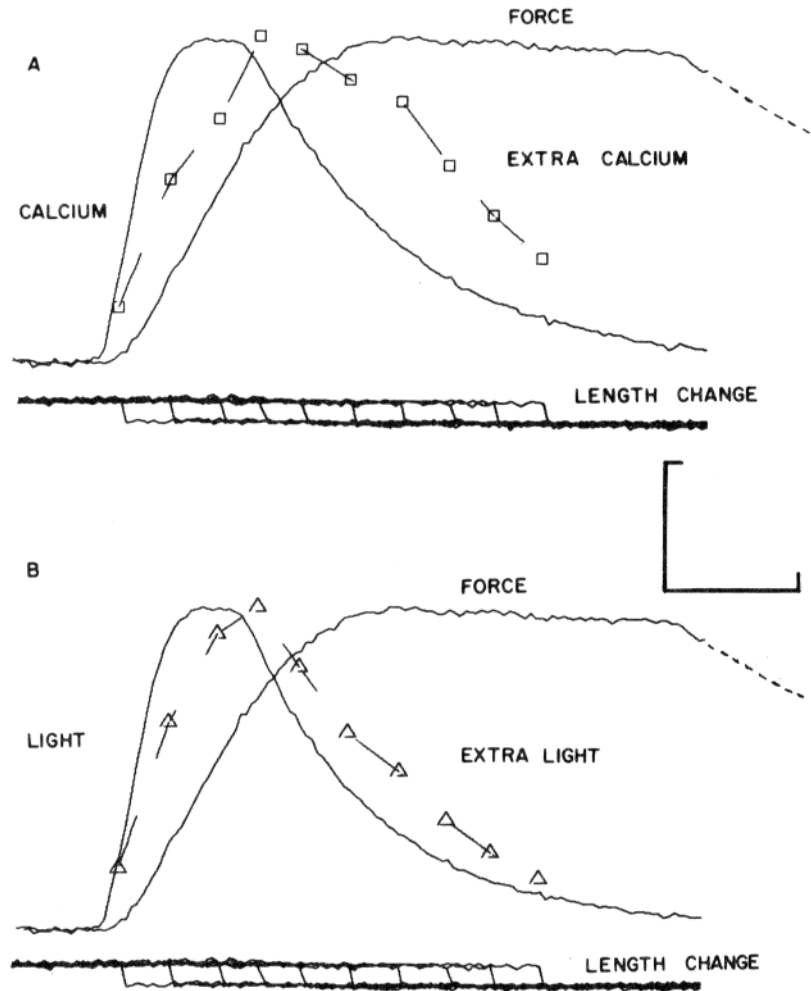


FIGURE 15. Time course of extra calcium. (A) Calculated relative free calcium and extra calcium; (B) aequorin light signal and extra light signal from which relative free calcium and extra calcium are derived. Traces: relative free calcium calculated from a control aequorin light signal with no length change using Eq. 1 and scaled to have the same amplitude as force; relative extra calcium calculated from the extra light signal using Eq. 2, scaled (by $10.8\times$) to have the same relative amplitude as the calcium curve and plotted as occurring at the time of the length change (squares, broken line); control isometric force at 3.8 g/cal with relaxation dotted in from a separate record taken with a slower time base; the length change was 0.5 mm for each shortening step; a control aequorin light signal with no length change at 400 nA/cal ; the extra light on release at 47 nA/cal plotted as occurring at the time of the length change (triangles, broken line). Except for the length steps, all curves have been scaled to the same maximum amplitude to allow better comparison of relative time courses. Horizontal sweep, 400 ms/cal ; data are taken from records similar to those shown in Fig. 13. Fiber length, 21 mm ; fiber weight, 45 mg ; temperature, 7.5°C ; resting glow, 200 nA .

The various intracellular pools are: (a) calcium sequestered in the sarcoplasmic reticulum, which could produce this phenomenon either by increased release from the reticulum or a decreased rate of uptake; (b) calcium bound to the contractile proteins; and (c) calcium bound to other calcium binding sites within the muscle.

The extra calcium is probably not due to a decreased calcium uptake by the sarcoplasmic reticulum since: (a) as shown in Fig. 13, *B-D*, shortening does not merely decrease the rate of decline of the calcium transient; it actually reverses the decline and increases the net calcium so that extra calcium is mobilized from some location as a result of the length change; (b) the time constant of the decline of the calcium transient (which probably, in part, reflects Ca accumulation by the sarcoplasmic reticulum) is not sensitive to muscle length (see Figs. 2 and 6) over the range of lengths involved (for example, the fiber in Figs. 2, 4, and 13 showed a <1% variation for all the lengths tested, with no systematic changes); (c) if there were a decrease in the rate of accumulation of calcium by the sarcoplasmic reticulum, one would expect additional force to be produced, whereas less is observed (see Figs. 2 and 4).

Evidence against the hypothesis that the extra calcium is being released from the sarcoplasmic reticulum comes from several observations.

(a) At the time when the post-stimulus length change produces extra light, the membrane potential has been repolarized to the resting level. Repolarizing the membrane to a value more negative than the resting level does not change the calcium transient (see Fig. 8). Thus, the membrane potential is under reasonable control and the muscle acts as if the membrane potential were back to its resting level at this time. Comparable length changes in a resting muscle do not produce extra light (Fig. 6). Consequently, we believe that although we cannot specify the mechanism of E-C coupling, we can nevertheless control E-C coupling with the voltage clamp.

(b) We have shown (Gordon and Ridgway, 1976) that when the membrane is stimulated by a constant current rather than a constant voltage, the length-dependent calcium release seen in barnacles occurs through changes in membrane resistance and is such that shortening of the muscle causes less calcium release. Therefore, the length dependence of the calcium release, if present, would subtract from (i.e., reduce, not magnify) the extra light seen in the present experiments.

(c) In the two-pulse experiments shown in Figs. 11 and 12, less calcium was released by the SR with the same depolarization during the second pulse. We interpret this to mean that the sarcoplasmic reticulum's ability to release calcium has been compromised. But the same length change causes more extra calcium during the second response than during the first when done at a comparable free calcium level. Therefore, if one expected this extra calcium to be coming from the sarcoplasmic reticulum, one would predict less calcium during the second pulse when the SR's ability to release calcium was compromised, not more calcium, as observed.

(d) Recently, Stephenson and Williams (1982) reported that they observed extra light signals in skinned, detergent-treated (presumably SR-inactivated),

slow-twitch mammalian muscle on release and they observed decreased light on stretch.

The evidence in favor of the extra calcium coming from binding sites on the myofilament is as follows: (a) The amount of extra calcium seen is greater when the muscle force is higher, either by increased stimulus intensity (Fig. 10) or by summation of contraction (Figs. 11 and 12). Since both of these maneuvers should increase the level of calcium activation, and hence the level of bound calcium, this evidence suggests that the extra light follows the bound calcium. (b) The amount of extra calcium seen correlates well with the redevelopment of force seen after the release (Fig. 14). This would be expected if the length change sampled the calcium on the myofilaments and the calcium on the filaments determined how much force would redevelop. Because the releases plotted here were done late in the calcium transient when the free calcium is relatively low, little calcium can rebind to the filaments to produce subsequent activation, and therefore the subsequent redevelopment of force would depend upon what is found at the time of release. (c) The amount of extra calcium seen on a given release has a time course that is intermediate between the free calcium and force (Fig. 15), which is what would be predicted for calcium bound to the myofilaments (see, e.g., Robertson et al., 1981; Gillis et al., 1982).

Our experiments do not rule out the possibility that the calcium comes from binding sites in the muscle other than the myofilament sites, for example, from a soluble site. It is difficult to propose a mechanism to couple the length change to a soluble site. It is also difficult to propose a mechanism for this which would correlate well with the redeveloped force, the time course, and the increase with additional force in the muscle, as well as not correlating with the force in the muscle at the time of the release. This does not entirely eliminate calcium bound to a soluble site as a source, but it makes it highly unlikely.¹

If the extra calcium is coming from the myofilaments, the question arises as to which of the various calcium binding sites are involved. There is also some question in the barnacle muscle about whether there is dual regulation, with calcium binding both to thin and thick filaments. Lehman and Szent-Györgyi (1975) propose this on the basis of their tests for localization of calcium sensitivity. Their values for the ATPase activity controlled through thick-filament regulation are very low, so there may be some doubt about this phase of control. Even if the activation is only through one of the sets of filaments, once calcium binds to a protein, there could be a number of intermediate states formed during this association, so that the calcium that we are sampling may represent just one of a

¹ After this article was initially submitted for publication, we were made aware of results from aequorin-injected frog muscle by Cecchi et al. (1984) that a shortening step made during the plateau of a tetanus produces a fall, not a rise, in the aequorin light signal. The difference between their results and ours may be accounted for by differences in the experimental conditions. Their results were obtained during stimulation, under action potential conditions, at tetanic force levels. Our results are post-stimulus, under voltage-clamp conditions, at subtetanic force levels. If we perform length changes during a long-duration stimulus without voltage control in the barnacle, we see variable results which only become consistent if voltage control is imposed. We have not done experiments at tetanic force levels because of the risk of breaking the ligature holding the fiber to the cannula.

number of intermediates between calcium binding and force production. We are not suggesting that the length change samples all of the bound calcium, but only that the length change samples an intermediate, which is important for force production since redeveloped force depends so strongly on the amplitude of the extra calcium that we detect (Fig. 14).

The mechanism whereby the length change samples the bound calcium is not clear at present. It could be that the length change detaches cross-bridges decreasing the calcium affinity of the thin- or thick-filament binding sites (possibly by increasing the rate of dissociation), releasing some calcium from those sites. The effects of stretch could be explained if stretching cross-bridges increased calcium affinity and produced increased calcium binding. A more detailed discussion requires further information about the factors that affect the extra calcium. We have used the term "sampling the calcium bound to an intermediate state," but, of course, we are assuming that the length change affects this intermediate to the same extent throughout contraction, a hypothesis that has not been tested.

The data shown in Fig. 15 have a number of implications for the mechanism of activation of contraction. A major one is that the calcium affinity of a binding site, presumably on the myofilaments, is sensitive to cross-bridge interaction, muscle length, force, or some combination of these. In other words, the mechanical state of the muscle can affect the calcium binding to the myofilaments. This offers a feedback mechanism between the state of contraction and the state of activation. It implies interaction between muscle force and muscle activation, which could have an important effect on activation and show up in such properties as the tension-pCa relationship, which contributes to the apparent cooperativity (Brandt et al., 1980; Ridgway et al., 1983).

A second implication concerns the time course of the intermediate. The amount of calcium bound to this intermediate rises after the rise in free calcium level, but some 100 ms before the rise in force. The precise timing depends to a great extent on the fidelity of the mechanical system used to record the rise of force. Since our system is not ideal, this estimate of the delay is only approximate. The delay between the rise of this intermediate and the rise of force may be attributable to conformational changes that must occur after the binding of calcium or the formation of this intermediate. It could also reflect the slow rate of attachment of cross-bridges and force production after calcium activation, and any internal shortening that might occur as well.

A third implication of the data shown in Fig. 15 is that there is a substantial delay between the decline of free calcium concentration and the decline of the calcium bound to the intermediate. This implies a slow OFF rate of calcium dissociation from the binding sites. A similar conclusion regarding the time course of the bound calcium has been reached by Baylor et al. (1982*b*) from experiments on frog muscle. The calculations done by Robertson et al. (1981) for mammalian muscle, based on their measured rates of calcium interaction with troponin and other calcium binding proteins, and those of Gillis et al. (1982) using the same rate constants plus a variable amount of parvalbumin, imply a much more rapid rate of dissociation. It is not known what the dissociation rate

would be for calcium binding sites in barnacle or, for that matter, even whether they are on troponin. In any case, the delay shown in Fig. 15A is some 10 times slower than that described by Robertson et al. (1981). Part of this difference could be accounted for by the fact that their calculation was for 20°C, while our measurements are made at ~8°C. Another possible origin of difference is that they assumed a fixed value for the OFF rate for calcium binding for troponin. This study implies that there is an effect of force (or length) on calcium binding (presumably through changes in the OFF rate; see also Ridgway et al., 1983), with increasing force increasing calcium binding. These factors could produce an increased lag between the decline of free calcium and force. Also, barnacle muscle is slower than frog muscle in shortening speed, so this may not be surprising. Whether it is a direct molecular difference in the calcium binding site or some property of protein interaction is not understood.

The long delay between the decline of the calcium transient and the decline of force has led to the development of several models of calcium activation (Ridgway, 1969; Ashley and Moiescu, 1973; Luttgau and Moiescu, 1978). There are two extremes. The "fast" calcium hypothesis is that calcium binds rapidly to the contractile filaments and returns to the sarcoplasmic reticulum with the time course of the decline of the calcium transient, with activation being maintained beyond that time by changes in the myofilaments. The "slow" calcium hypothesis is that free calcium is elevated during activation, with the binding to the myofilaments accounting for the decline of the calcium transient. During the rest of contraction and relaxation, the calcium remains bound to the myofilaments coming off slowly during relaxation. Our data, shown in Fig. 15, imply that barnacle muscle operates somewhere between these two, probably being closer to the fast than the slow hypothesis. There appears to be a substantial period during which force is produced in the muscle while the free calcium and the calcium bound to the myofilaments are both reduced to low levels. As would be predicted from many kinetic models (see Julian, 1969, for example), the decline of the filament-bound calcium and the decline of force are not necessarily related, because the decline of force depends upon the breaking of attached cross-bridges, which, according to our model, occurs sometime after the removal of calcium. Although the myofilaments may be more sensitive to calcium during relaxation (Ridgway et al., 1983), and there may be "tails" on the calcium transient (Ashley and Lignon, 1981), we believe that force is sustained by cross-bridge interaction and not by calcium.

Relaxation is enhanced by manipulations that detach cross-bridges. This was seen very clearly to be the case by Huxley and Simmons (1970), who discovered that the phase of rapid relaxation from a tetanus in frog muscles occurred when part of the muscle lost its activation and was stretched, allowing the still active portions to shorten rapidly. The shortening-induced detachment of cross-bridges can decrease muscle force in at least three ways. First, shortening-induced detachment breaks bridges more rapidly than in an isometric relaxation (Huxley and Simmons, 1970). Second, as shown in Fig. 2, shortening-induced detachment presumably frees calcium bound to the myofibrillar-activating sites, possibly by changing the calcium affinity (Bremel and Weber, 1972). Third, shortening-

induced detachment also removes the possible cooperative role that attached cross-bridges may have, which allows more cross-bridges to attach both in the presence and absence of calcium (Murray and Weber, 1981). Each of these three factors tends to cause a decrease in muscle force with shortening and could, separately or together, contribute to the shortening-induced "deactivation" (Edman, 1975). The relative contribution of each of these factors to deactivation remains to be determined.

In conclusion, the properties of the extra calcium seen with post-stimulus length changes suggest: (a) that the calcium binding to the activating myofibrillar proteins is sensitive to muscle length or muscle force, and (b) that there is a pool of bound calcium that has a time course intermediate between the free calcium concentration and force.

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