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CALCIUM CONCENTRATION IN THE MYOPLASM OF SKINNED FERRET VENTRICULAR MUSCLE FOLLOWING CHANGES IN MUSCLE LENGTH

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SUMMARY

1. Ferret ventricular muscles were skinned by prolonged application of Triton X-100. Aequorin was allowed to diffuse into the myoplasmic space and the resulting light emission was used to monitor the myoplasmic [Ca^{2+}]. The muscle was then activated with a lightly buffered Ca^{2+} solution and the changes in myoplasmic [Ca^{2+}] and tension in response to length changes were investigated.

2. A sudden reduction in muscle length led to a rapid increase in myoplasmic [Ca^{2+}] to a new level which was maintained as long as muscle length was reduced and which was reversed when the muscle was stretched back to the control length. The rate of increase of [Ca^{2+}] when the muscle length was reduced was greater than the rate of decrease in [Ca^{2+}] when the muscle was stretched.

3. Increasing the concentration of EGTA in the activating solution, so as to increase its Ca^{2+}-buffering capacity, eliminated the changes in myoplasmic [Ca^{2+}] in response to a length change but had little effect on developed tension.

4. On stretching the muscle there was a slow component of recovery of tension with a time course broadly similar to the rate of decrease of myoplasmic [Ca^{2+}]. The time course of tension redevelopment and of the accompanying reduction in myoplasmic [Ca^{2+}] both decreased to a similar extent when the [Ca^{2+}] used to activate the muscle was increased.

5. Step reductions of length of increasing amplitude caused increases in myoplasmic [Ca^{2+}] which were larger in proportion to the size of the step.

6. Step reductions of length of equal size but from different starting lengths caused changes in myoplasmic [Ca^{2+}] the amplitude of which correlated with the change in tension rather than the change in length.

7. The increase in myoplasmic [Ca^{2+}] when muscle length is reduced suggests that Ca^{2+} is released from a site in the muscle, probably troponin C. The time course and magnitude of the changes in myoplasmic [Ca^{2+}] correlate more closely with the changes in developed tension than muscle length.

INTRODUCTION

The tension developed by cardiac muscle depends on the length of the muscle; this property underlies Starling's Law of the Heart (Patterson & Starling, 1914) and

plays an important role in the response of the heart to exercise and to cardiac failure (Braunwald & Ross, 1979). The cellular mechanisms which lead to the length–tension relation in cardiac muscle are not established though it is now clear that changes in myofilament overlap (Gordon, Huxley & Julian, 1966) make only a small contribution (for reviews, see Jewell, 1977; Allen & Kentish, 1985*a*; Lakatta, 1986). There is considerable evidence that activation of the contractile proteins by Ca^{2+} varies with muscle length (Allen, Jewell & Murray, 1974; Fabiato & Fabiato, 1975). This could come about either by changes in Ca^{2+} release from the sarcoplasmic reticulum or by changes in the sensitivity of the contractile proteins to Ca^{2+}. There is evidence for the operation of both processes (Allen & Kurihara, 1982; Hibberd & Jewell, 1982).

In the present study we are concerned with the mechanism by which changes in muscle length lead to alterations in the Ca^{2+} sensitivity of contractile proteins. The evidence for this change in Ca^{2+} sensitivity comes from two kinds of experiments. Intact muscles injected with the Ca^{2+}-sensitive protein aequorin show an increase in myoplasmic [Ca^{2+}] when the muscle is suddenly shortened, suggesting that Ca^{2+} has been released from a binding site (Allen & Kurihara, 1982; Housmans, Lee & Blinks, 1983; Gordon & Ridgway, 1987). However the interpretation of these experiments is complicated by the possibility that Ca^{2+} release from the sarcoplasmic reticulum or Ca^{2+} entry across the surface membrane may be affected by muscle length. More direct evidence comes from experiments with skinned cardiac preparations, with their surface membrane destroyed, in which an increase in muscle length over the physiological range leads to an increase in the apparent Ca^{2+} sensitivity (Hibberd & Jewell, 1982; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986). Two possible mechanisms for the increase in apparent calcium sensitivity are (i) an increase in the amount of Ca^{2+} bound to troponin at a given [Ca^{2+}] and (ii) an increase in the effectiveness with which a given amount of Ca^{2+} bound to troponin turns on cross-bridge activity. The present study was designed to distinguish between these possibilities by measuring myoplasmic [Ca^{2+}] when muscle length was changed. The experiments were performed on skinned cardiac muscle in which all membranes had been destroyed by detergent, leaving troponin C (TnC) as the major site of Ca^{2+} binding (Pan & Solaro, 1987). Aequorin was allowed to diffuse into the myoplasmic space to detect any changes in [Ca^{2+}] when muscle length was changed; such changes in [Ca^{2+}] would only be expected if the affinity of TnC for Ca^{2+} was affected by muscle length.

Some of these results have been presented in preliminary form (Allen & Kentish, 1985*b*).

METHODS

Apparatus

Two muscle baths were used. One, used for aequorin loading, was a 35 μl well made of plastic tubing. The other, used for the remainder of the experiments, consisted of two horizontally mounted microscope cover-slips 1 mm apart. Solutions could flow through the bath by means of an opening at one end, connected to a non-pulsatile pump, and an opening at the other, connected to a suction line. Both baths were mounted on a spring-loaded base; by depressing the base and moving it along, the bath surrounding the muscle could be changed in 1–2 s. Isometric tension was measured with an AME 801 silicon beam with a carbon-fibre extension arm. Muscle length could be altered with an electromagnetic lever (Cambridge 300S), driven by a ramp generator. Light from

aequorin inside the muscles was measured using a photomultiplier tube with a methylacrylate light guide (diameter 10 mm), which could be brought to within 1 mm of the muscle. The entire apparatus was housed in a light-tight box. The box was closed before the PMT shutter was opened. Muscle length, tension and aequorin light were recorded continuously on a chart recorder and FM tape. Responses to length changes were averaged later.

Light signals were low-pass filtered (bandwidth 0–100 Hz) to reduce photon noise. Tension and length signals were subject to the bandwidth of the tape recorder (bandwidth 0–300 Hz).

Experimental procedure

The procedures for mounting and for skinning the muscles were similar to those previously described (Kentish, 1986). Trabeculae or thin papillary muscles were dissected from the right or left ventricles of ferrets (or, in a few experiments, rats). The preparation was then mounted in the flow-through muscle bath using snares of gold-plated tungsten wire to attach the muscle to the tension transducer and motor. The muscles used in this study were 100–350 μm wide and 1–2·5 mm long. Once the muscles were mounted, as much as possible of the damaged ends beyond the snares was dissected away. Initially the muscles were bathed in Tyrode solution and were stimulated. The muscle length was increased until maximum developed tension was obtained (L_{max}). In the few muscles where the resting sarcomere length could be measured by laser diffraction (in most muscles the diffraction pattern was too diffuse), L_{max} corresponded to a sarcomere length of about 2·2 μm, in agreement with earlier work (Julian & Sollins, 1975). The muscles were then skinned by perfusion for 1–2 h with relaxing solution (see below) to which had been added 1% Triton X-100. The sarcomere length did not change appreciably during the skinning procedure. The skinning period was longer than used previously (Kentish *et al.* 1986) in order to ensure complete disruption of the sarcolemma and sarcoplasmic reticulum. In some experiments this was carried even further by skinning the muscles for 24 h at 4 °C in the Triton solution. There was no obvious difference between muscles skinned for 24 h and those skinned for 1–2 h.

All experiments were done at room temperature (20–22 °C). The solutions used during and after the skinning procedure had the following basic composition (mM): 40 potassium proprionate, 100 BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid), 5 MgATP, 3 Mg^{2+}, 1 dithiothreitol, 10 sodium creatine phosphate, pH 7·0, plus EGTA or CaEGTA to produce a range of free Ca^{2+} concentrations from 1 nM (relaxing solution) to 200 μM (maximal activating solution). The final ionic strength was 170 mM. To aid the detection of Ca^{2+} release from the myofilaments, the solutions were only weakly buffered with EGTA (100 μM). The contaminant Ca^{2+} (20 μM) and the EGTA purity were determined as previously described (Kentish, 1984).

In some experiments, in order to eliminate the changes in myoplasmic [Ca^{2+}], EGTA concentration was increased to 1 mM. A possible source of concern under these circumstances is whether EGTA is capable of binding Ca^{2+} sufficiently rapidly to prevent the changes in myoplasmic [Ca^{2+}]. To address this point we have calculated the time constant of change of [Ca^{2+}] when total EGTA = 1 mM, free [EGTA] = 27 μM, [Ca^{2+}] = 15 μM. Using the equation given by Thomas (1982, p. 47) and a rate constant of binding of Ca^{2+} to EGTA of 2×10^6 M^{-1} s^{-1} and an off rate constant of 0·4 s^{-1}, we calculate the time constant of changes in [Ca^{2+}] to be 12 ms. Thus a 1 mM-EGTA buffer is capable of binding Ca^{2+} sufficiently rapidly to greatly reduce the changes in [Ca^{2+}] which we observed in the more weakly buffered solutions.

Ca^{2+}-release experiments

After skinning was completed, the muscle was bathed in relaxing solution in the flow-through muscle bath for at least 30 min to wash out the Triton. The muscle was activated with a maximal activating solution (200 μM-Ca^{2+}) several times. The muscle was then transferred to the 35 μl bath containing relaxing solution (100 μM-EGTA) with aequorin (15 μM). This solution was stirred by slowly stretching and releasing the muscle. The muscles were bathed in this solution for 10 min to allow the aequorin to diffuse into the myoplasmic space of the preparation (cf. Ashley, Moisescu & Rose, 1974). This period was sufficient for the aequorin to equilibrate with the muscle, as judged by the magnitude of the light response when the muscle was subsequently exposed to Ca^{2+}.

After the muscle was loaded with aequorin, the muscle was transferred to the flow-through bath containing the desired activating solution (1–200 μM-Ca^{2+}, 100 μM-EGTA). The PMT light-guide was then moved up as close as possible to the muscle (< 1 mm) and the PMT shutter was opened. Aequorin light and muscle tension increased as Ca^{2+} entered the muscle (Fig. 1). Once tension

in the contracture was steady, the muscle was subjected to releases and stretches of various magnitudes and time courses. When the desired length changes or solution changes had been completed, relaxing solution (1 mM-EGTA) was pumped into the bath. After a few minutes the muscle was returned to the aequorin-loading bath and the whole procedure repeated. The aequorin light on subsequent loadings was variable so that it was necessary to compare interventions within a single loading period to make quantitative comparisons.

These experiments depend upon the fact that aequorin diffuses out of the muscle considerably more slowly than Ca^{2+} and CaEGTA diffuse into the muscle (cf. Ashley *et al.* 1974). We found that the diameter of the muscle was important and it was necessary to use muscles of a diameter larger than optimum for the rapid control of the myoplasmic ionic concentrations (see Discussion in Kentish & Jewell, 1984). In smaller preparations (diameter less than about 200 μm), the aequorin signal declined very rapidly and responses to length changes were small. This is probably because Ca^{2+} released near the edge of the preparation could diffuse rapidly into the very large volume of fluid outside the muscle where it would be buffered. This effect is minimized in large preparations.

One problem encountered in the early stages of this study was that tension development decreased substantially in successive exposures to the activating solutions. This excessive deterioration of the muscle was thought to be due to evaporation of water from the aequorin-loading bath (indicated by an increase in the activity of aequorin), causing an increase in ionic strength to levels at which appreciable deterioration of the muscle occurs (Kentish, 1984). To minimize this problem the aequorin bath was surrounded by pieces of wet tissue and water was added to the bath at intervals to keep the solution meniscus at a constant level.

Interpretation of light measurements

It is important to establish that the efficiency of aequorin light collection did not vary with muscle position since this could lead to artifactual changes in light collection when muscle length was changed. Bearing in mind the length of the muscles (1–2·5 mm) and the diameter of the light guide (10 mm), one would not expect to see a variation in the efficiency of light collection with movement of the muscle. This assumption has been tested previously (Cannell & Allen, 1983) using a cylindrical light source (diameter 1 mm, length 2 mm) at the same position relative to the light guide as the muscle in the present experiments. These tests showed that movements along the axis of the light source of $\pm$ 1·5 mm had no detectable effect on light collection. The muscles used in the present study were comparable in size to the light source used above and the greatest movements were 0·5 mm, so there should have been no variation in light collection with position or shortening. The observation that changes in light signal were abolished by increasing the EGTA concentration (see Results and Fig. 3) confirm this conclusion.

All the experimental figures show unconverted aequorin light signals. To convert changes in aequorin light to changes in $[Ca^{2+}]$, we have used the relation between aequorin light (L) and $[Ca^{2+}]$ described by Allen, Blinks & Prendergast (1977). To a good approximation, over the range of $[Ca^{2+}]$ used in this study, $L = k([Ca^{2+}])^{2.5}$ so that if light increases from L_1 to L_2 the change of $[Ca^{2+}]$ is $[Ca^{2+}_2]/[Ca^{2+}_1] = (L_2/L_1)^{0.4}$.

One complication of the present study was that not only did the aequorin light emission decline following aequorin loading and the application of high Ca^{2+} but, in addition, the ratio of light change during a release to the light level preceding the release ($\Delta L/L$ or fractional light change) also showed a decline (Figs 5*A* and 6*A*). One possible cause for the decline in this ratio was that a part of the total light emission came from regions of the preparation which were not influenced by length changes, and that this component of light decayed more slowly than the light from regions that were subject to length changes. The cut ends of the preparation (i.e. those parts extending beyond the tip of the transducer or motor arm) and damaged muscle just inside the snares could make a contribution of this sort. In some experiments the contribution of light emission from the ends outside the snares was minimized by painting them with quick-drying black paint. To minimize the effects of changes in the ratio light signal, whenever possible, control records were taken both before and after test records and the control record shown in the figures is averaged from controls both before and after the test period. Unfortunately changes in the activating $[Ca^{2+}]$, because they required 1–2 min to equilibrate throughout the preparation, took too long to allow a complete cycle of control, new $[Ca^{2+}]$, followed by control. For this reason we were unable to test the effects of $[Ca^{2+}]$ on the amplitude of the light changes induced by muscle length in a systematic way.

RESULTS

Figure 1 shows the changes in tension and aequorin light after a skinned muscle was transferred from the 35 μl bath containing aequorin to the flow-through bath containing 15 μM-Ca^{2+}. As the Ca^{2+} diffused into the muscle the aequorin light and tension development rose, though only slowly because of the low Ca^{2+}-buffering power of the solution. Tension reached a steady level in about 2 min but the aequorin

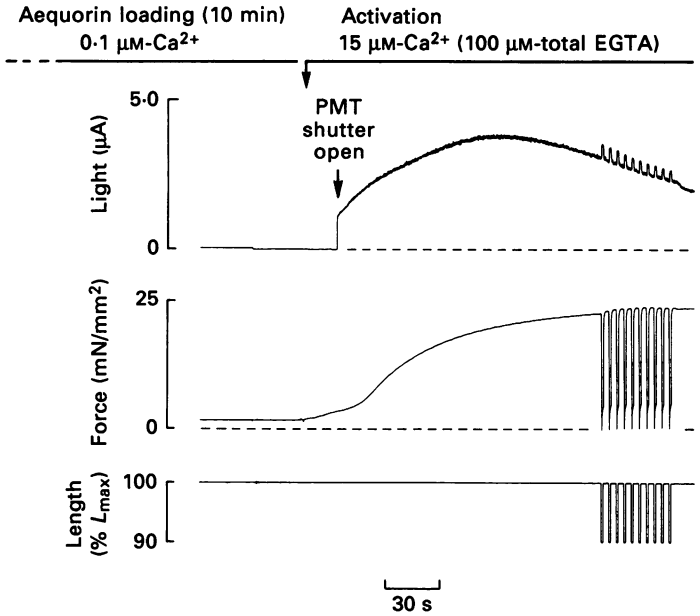

Fig. 1. Apparent release of Ca^{2+} induced by shortening in a detergent-skinned trabecula. Traces of aequorin light emission (top), muscle tension (middle) and muscle length (bottom). The muscle was loaded with aequorin in relaxing solution for 10 min, then was placed in activating solution of 15 μM-Ca^{2+}, which produced 80% of maximum tension. Shortening the muscle by 10% (for 1 s every 4 s) produced a fall of tension and an increase of aequorin light.

light fell after reaching a peak at 1–2 min. This fall of light was probably the result of two processes: (i) aequorin consumption by Ca^{2+} entering the muscle and (ii) aequorin diffusion out of the muscle (the aequorin would then be carried out of range of the light guide of the photomultiplier tube by the flow of solution). When tension was steady the muscle was shortened rapidly (within 10 ms) by 10% of its initial length (L_{max}) and held at the new length for 1 s every 4 s. In this muscle, each release of the muscle reduced muscle tension by about 90%. In addition, the release caused an increase in the light emitted by aequorin. In five muscles, a 10% reduction in muscle length caused light to increase by 11·4 ± 2·4% (mean ± S.E.M.). All the results illustrated are from ferret muscles but we observed similar increases in light on shortening in several rat preparations. The increase in aequorin light shows that the $[Ca^{2+}]$ in the myoplasmic space within the preparation had increased; since troponin

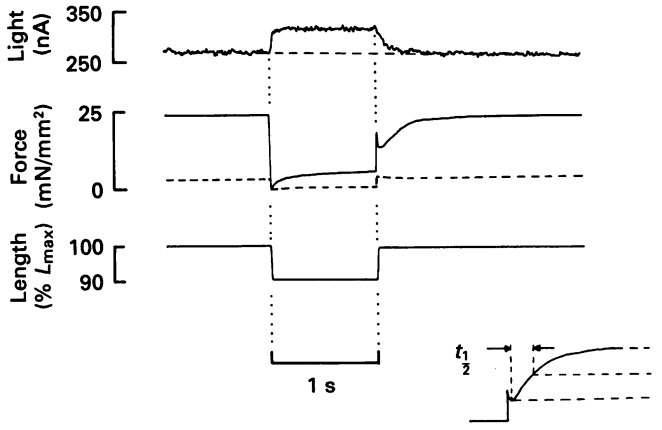

Fig. 2. Averaged records ($n = 8$) from Fig. 1 on a faster time scale. The traces show aequorin light (top), tension (middle) and muscle length (bottom). The small downward slope in the top trace (dashed line) represents the consumption and loss of aequorin as seen in Fig. 1. The dashed line in the middle trace shows the changes in resting tension when the muscle length was altered at $[Ca^{2+}] < 100$ nM. The $[Ca^{2+}]$ during activation was 15 μM. Inset (lower right) indicates the method used to measure the $t_{\frac{1}{2}}$ of the slow phase of recovery of tension.

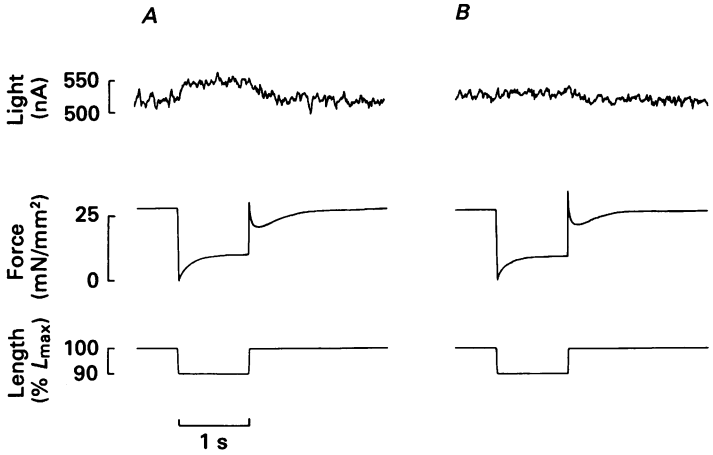

Fig. 3. Light and tension responses to a 10% shortening of a muscle at two concentrations of EGTA: 0·1 mM (*A*, control) and 1·0 mM (*B*). The total Ca^{2+} concentration was adjusted to produce 15 μM-free Ca^{2+} in each case. At the higher [EGTA] the light response was diminished but the tension responses were not changed appreciably. Averaged records ($n = 8$).

C is the major Ca^{2+} buffer within a skinned preparation (Pan & Solaro, 1987), the increase in $[Ca^{2+}]$ suggests that shortening causes Ca^{2+} release from TnC.

The results in Fig. 1 are shown in Fig. 2 on a faster time scale. These averaged records show that when muscle length was decreased by 10%, tension fell to almost zero and then recovered partially. A small part (10–20%) of the decrease in tension was due to the reduction in passive tension in the muscle (dashed line, Fig. 2). Most of the large decrease in tension was probably not due to changes in Ca^{2+} activation,

but to mechanical factors, such as presence of substantial internal restoring forces; in these preparations the tension–length relationship was quite steep even when the myofibrils were maximally activated by Ca^{2+} (unpublished observations). Light increased rapidly ($t_{\frac{1}{2}} \approx 15$ ms) by about 20% and stayed constant for the period the muscle length was held short. The apparent time constant of about 15 ms for the rise in light should not have been limited by the filter used (100 Hz, $t_{\frac{1}{2}}$ 1 ms) or by the response time of aequorin ($t_{\frac{1}{2}}$ 7 ms).

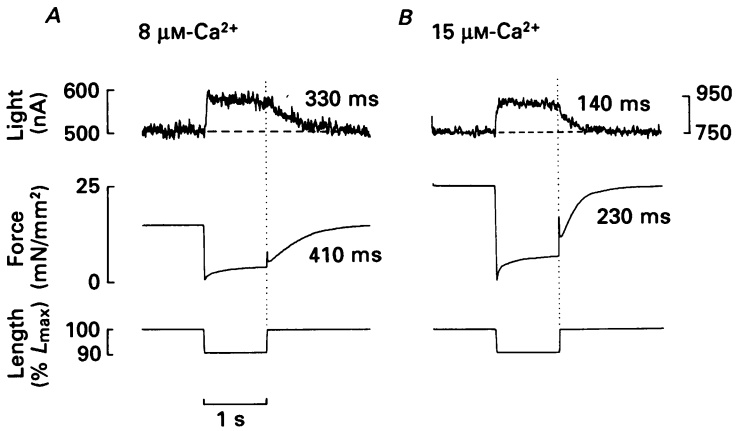

Fig. 4. Light and tension responses to a length change at two concentrations of free Ca^{2+}: 8 and 15 μM. Note the different calibrations for light in the two panels. The half-times for the changes in light and tension after the increase in length are shown next to the traces. (For the light records, the $t_{\frac{1}{2}}$ was measured as half the time taken for light to change from one steady value to another; for tension, see Fig. 2.) Averaged records ($n = 8$).

This 20% increase in light is equivalent to a rise in $[Ca^{2+}]$ of about 8%; that is, the $[Ca^{2+}]$ inside the muscle rose from 15 to 16 μM. When the muscle was restretched to L_{max}, the tension response showed a rapid transient increase, part of which was passive (dashed line), then a small rapid decline, followed by a slow secondary increase of tension over 1 s. This slow redevelopment of tension following a length increase is a pronounced feature of cardiac muscle (Steiger, 1975). It is thought to be caused by slow net reattachment of cross-bridges because stiffness, a measure of the number of attached cross-bridges, rises in parallel with tension (Herzig & Ruegg, 1977). After the restretch, light also returned towards its original level but slowly ($t_{\frac{1}{2}} \approx 75$ ms). Note that the length change was rapid (10 ms) and similar on both release and stretch. When the muscle was shortened both the rise of light and the decline of tension were rapid ($t_{\frac{1}{2}} < 15$ ms). On the other hand when the muscle was stretched both the slow phase of recovery of tension ($t_{\frac{1}{2}} \approx 175$ ms) and the decline of light ($t_{\frac{1}{2}} \approx 75$ ms) were slow. Other examples of this disparity can be seen in Figs 3–6. (The method used to estimate the $t_{\frac{1}{2}}$ of tension recovery is shown in the inset to Fig. 2. If the $t_{\frac{1}{2}}$ for tension recovery was measured in a different way, using the change in tension from one steady level to another, the $t_{\frac{1}{2}}$ values were reduced but the conclusions are the same.)

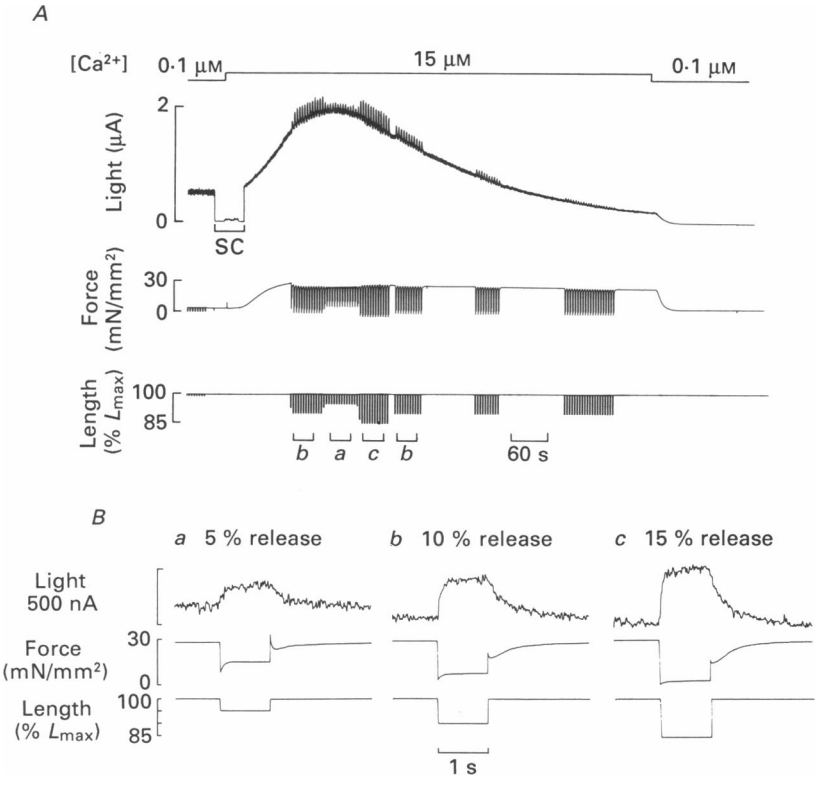

Fig. 5. Light and tension responses in a muscle shortened by 5% L_{max}, 10% L_{max} and 15% L_{max}. Panel *A* shows continuous records of light, tension and length. Panel *B* shows the averaged records of the same experiment. The periods used to obtain the averaged record are indicated on *A*. Each trace is the average from eight length changes, with the 10% trace consisting of four traces in the 1st series and four traces in the 2nd series. (SC, shutter closed.)

The time courses described above show that there is a closer correlation between tension and light than between length and light. This suggests that the process leading to changes in light may be more closely linked to tension than to muscle length itself. On the hypothesis outlined above, it could be suggested that the Ca^{2+} release following shortening is a consequence of the fall of tension and that the slow decrease of light upon muscle restretch is because the redevelopment of tension is slow.

Effect of an increase of EGTA concentration

It is important to establish whether the slow recovery of tension after a stretch is the cause or the consequence of the slow decrease in myoplasmic [Ca^{2+}] which occurs over the same period. To this end we increased the level of EGTA from 0·1 to 1 mM to increase the level of Ca^{2+} buffering and thereby minimize the changes in myoplasmic [Ca^{2+}] without changing the mean level of myoplasmic [Ca^{2+}]. If the changes in tension were a consequence of the changes in [Ca^{2+}] this should reduce the changes in tension. Figure 3 shows that increasing the [EGTA] (at constant [Ca^{2+}])

produced a considerable reduction of the light response, while the magnitude and time course of tension changes were hardly affected. Thus it appears that the changes in tension are independent of the changes in myoplasmic $[Ca^{2+}]$ which the length changes produce. This experiment also provides evidence that the changes in $[Ca^{2+}]$ are not artifacts associated with movements of the muscle. If this were the case the changes in light would not have been eliminated simply by increasing the Ca^{2+} buffering within the muscle.

Influence of $[Ca^{2+}]$

To test the association between the time courses of tension recovery and the change in $[Ca^{2+}]$ further, we varied the $[Ca^{2+}]$ in the activating solution so as to alter the rate of tension recovery. Figure 4 shows the results obtained using 8 μM-Ca^{2+} and 15 μM-Ca^{2+}. Note that in 8 μM-Ca^{2+} the steady tension at L_{max} was half that in 15 μM-Ca^{2+}. As reported by Steiger (1975) and Herzig & Ruegg (1977), an increase in $[Ca^{2+}]$ accelerates the slow phase of tension redevelopment. The $t_{\frac{1}{2}}$ for the tension increase after restretch fell from 410 to 230 ms as the $[Ca^{2+}]$ was raised from 8 to 15 μM. The higher $[Ca^{2+}]$ led to a greater light emission and a greater fractional light emission but, as noted in the Methods, the quantitative significance of this is uncertain. However the time course of the changes in light are unaffected by this uncertainty and the increased activating $[Ca^{2+}]$ caused the $t_{\frac{1}{2}}$ to fall from 330 to 140 ms. Thus the light response varied in parallel with the change in tension, even though the length change was identical in the two cases.

As illustrated in Fig. 4, it was usually the case that the decrease in light had a faster time course than tension redevelopment. This was true even when light was converted to $[Ca^{2+}]$. For the responses in Fig. 4, the $t_{\frac{1}{2}}$ for the decrease in $[Ca^{2+}]$ was not significantly different from those calculated for aequorin light. (It might be supposed that this should not be so, given that light is a power function of $[Ca^{2+}]$, but in fact for a small change in light superimposed on a large light level, the relative change in light $\Delta L/L$ is $\approx \Delta[Ca^{2+}]/[Ca^{2+}]$; Baker, Hodgkin & Ridgway (1971)).

The magnitude of the change in length

A rapid (10 ms) shortening of muscle of > 1–2% of muscle length will lead to detachment of most cross-bridges. If this initial rapid cross-bridge detachment were the cause of Ca^{2+} release we would expect to observe a component of Ca^{2+} release which was independent of step size above 1–2% of muscle length. To investigate how the extent of Ca^{2+} release varied with the magnitude of the change in length, the muscle was shortened by different amounts (Fig. 5): by 5% L_{max}, 10% L_{max} or 15% L_{max}. Note that the 10% length change was repeated before and after the 5 and 15% changes; thus Fig. 5*B b* consists of four releases from the first period and four from the second. The responses to the different length changes were broadly similar and the changes in light and tension increased in parallel as the size of the length step was increased. This suggests that the amount of Ca^{2+} released was determined chiefly by the magnitude of the change in tension (or length) and was affected little by the sudden detachment of cross-bridges.

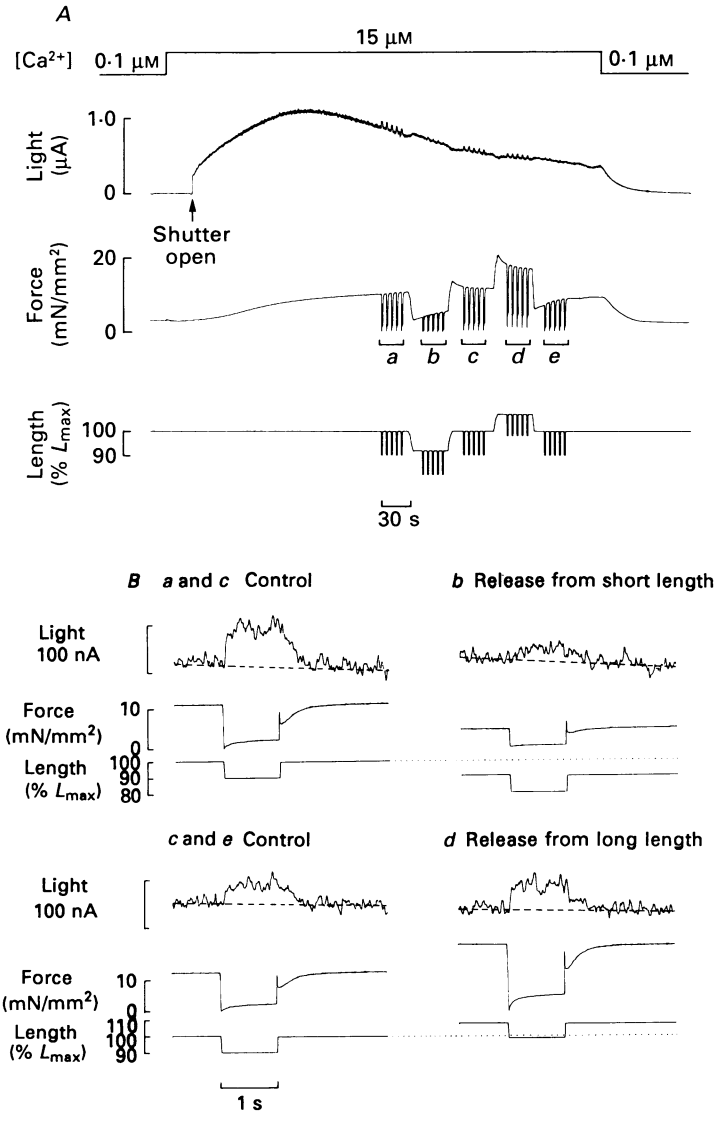

Fig. 6. Effect of shortening the skinned muscle by the same amount (9% L_{max}) but from different initial lengths. Panel *A* shows continuous records of the light, tension and length. Panel *B* shows the records on a faster time base averaged ($n = 4$) from the periods indicated on *A*.

Influence of initial muscle length

In the above experiment the size of shortening produced changes in $[Ca^{2+}]$ which were roughly in proportion to both the size of the length change and the change in tension. In a further attempt to distinguish the dependence of Ca^{2+} change on length or tension we used a constant shortening step but varied the initial length (Fig. 6). As in Fig. 5, the control periods of Fig. 6*B* were averages of releases from before and after the intervention to correct for slow changes in the light emission. As the

initial length was increased, steady tension increased (due to the length–tension relationship) and the absolute magnitude of the tension response was greater, even though the length change was the same. The same was true of the alterations of light, which were reduced when the muscle was released from the short length and which appeared to be increased with a release from the long length. Thus once again it seemed that the light responses were linked more closely with the tension changes than with the length changes.

DISCUSSION

It is likely that the steep length–tension relationship of cardiac muscle is at least partly due to a length dependence of the Ca^{2+} sensitivity of the cardiac myofibrils (Allen & Kentish, 1985*a*; Lakatta, 1986). This could occur in one of two ways. (i) Calcium binding to troponin could be affected by muscle length (or tension). (ii) The effectiveness of a given amount of Ca^{2+} bound to troponin in activating cross-bridge cycling could be affected by muscle length (or tension). Mechanism (i) predicts that changes in length should change the amount of Ca^{2+} bound to troponin and lead to changes in myoplasmic [Ca^{2+}]. Mechanism (ii) does not predict any such changes in [Ca^{2+}]. Our results clearly support mechanism (i) but they cannot confirm or refute the existence of mechanism (ii).

Previous measurements of Ca^{2+} binding were performed by measuring the total binding of $^{45}Ca^{2+}$ to muscles in rigor (Fuchs, 1977, 1978) or, more recently, muscles in ATP-containing solutions (Hofmann & Fuchs, 1987; Pan & Solaro, 1987). There is some discrepancy in these radiochemical studies, as Pan & Solaro (1987) reported no apparent change in Ca^{2+} binding to activated skinned papillary muscles at different sarcomere lengths, whereas Hofmann & Fuchs (1987) found that, in similar preparations in rigor, the affinity for Ca^{2+} increased as sarcomere length was raised. An additional problem, the significance of which has yet to be assessed, is that there is a slowly exchanging Ca^{2+} binding site which may complicate the interpretation of these radiochemical measurements (Pan & Solaro, 1987). Our method measures rapid changes in Ca^{2+} binding in Ca^{2+}-activated muscles and our results imply that the amount of Ca^{2+} bound *does* vary as muscle length is altered; this broadly agrees with the findings of Hofmann & Fuchs (1987).

The present study is complementary to those previously performed with intact cardiac and skeletal muscle fibres that had been microinjected with aequorin (Allen & Kurihara, 1982; Housmans *et al.* 1983; Gordon & Ridgway, 1987). The studies on intact fibres showed that release of the muscle during the later stages of the twitch produced a small upward inflexion on the falling aequorin light transient. These authors suggested that this inflexion was due to Ca^{2+} released from troponin as a result of the reduced muscle length, but in those experiments it was not possible to rule out the alternative that the Ca^{2+} came from non-myofibrillar structures such as the sarcoplasmic reticulum. In the present study, we used ferret papillary muscles, as used by Allen & Kurihara (1982), except that the sarcoplasmic reticulum and other membranous organelles were destroyed with detergent, thus precluding these as sources of the Ca^{2+} released. In support of this, Pan & Solaro (1987) found no evidence for other Ca^{2+} binding sites, such as those on the sarcolemma or sarco-

plasmic reticulum in Triton-skinned cardiac preparations. It therefore seems likely that, in the present study at least, the Ca^{2+} came from sites on the myofibril. The inference is that in the studies with intact cells, the Ca^{2+} released also came from the myofibrils rather than from organelles; this is supported by the close similarity between the two types of study with regard to the magnitude of the release; in both intact papillary muscles (Allen & Kurihara, 1982) and skinned papillary muscles (Fig. 2), a 10% decrease in muscle length caused light to rise by about 10%.

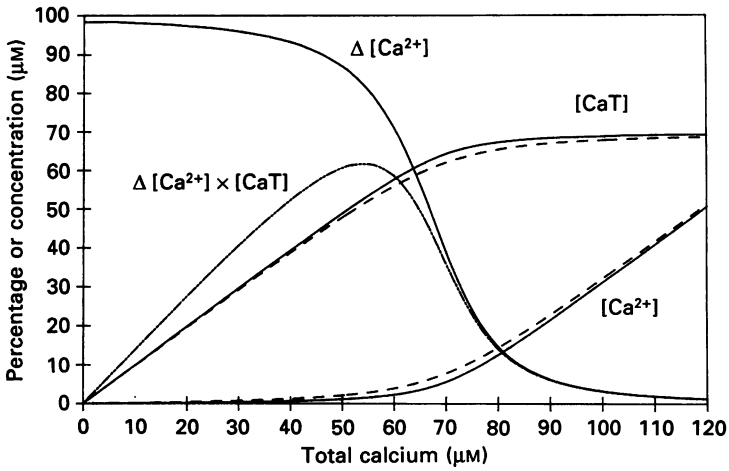

Fig. 7. Model of Ca^{2+} binding to troponin to illustrate the effect of changing the binding constant. Total [Tn] = 70 μM, total [Ca] variable, Ca^{2+} assumed to bind to only one site on troponin. [Ca^{2+}] and [CaT] refer to the concentrations of free Ca^{2+} and Ca^{2+} bound to troponin, respectively, when the binding constant (K) is 2×10^6 M^{-1} (continuous lines) or 1×10^6 M^{-1} (short dashed lines). Also shown are the percentage increase in [Ca^{2+}] (labelled Δ[Ca^{2+}]) produced when K is reduced by the factor of two, and the Δ[Ca^{2+}] (%) multiplied by the fractional occupancy of troponin, [CaT]/total [troponin] (labelled Δ[Ca^{2+}] × [CaT]; units %).

Source of the Ca^{2+} released

In the preceding discussion it has been assumed that the Ca^{2+} was released from TnC but it is necessary to exclude contributions from other Ca^{2+} binding proteins such as myosin light chains and actin. Our activating solutions contained a 3 mM-Mg^{2+}, at which the light chains of myosin bind negligible amounts of Ca^{2+} for solution [Ca^{2+}] below 10 μM (Holroyde, Robertson, Johnson, Solaro & Potter, 1980; Pan & Solaro, 1987). F-actin binds Ca^{2+} avidly, but the off-rate is far too slow (8×10^{-6} s^{-1}; Korn, 1982) to allow significant release of Ca^{2+} over the time scales studied here. Thus myosin light chains and actin are unlikely to contribute to the release of Ca^{2+}. This conclusion is supported by the fact that, in skinned muscles similar to those we used, Pan & Solaro (1987) found that the (steady-state) Ca^{2+} binding at [Ca^{2+}] < 10 μM could be entirely accounted for by binding to TnC. Thus it seems likely TnC was the source of the Ca^{2+} released. Furthermore, the site of Ca^{2+} release was probably the single low-affinity 'regulatory' Ca^{2+}-binding site on TnC, as the high-affinity Ca^{2+}–Mg^{2+} sites are unlikely to participate in the Ca^{2+} regulation of cardiac muscle because of their slow effective on-rates for Ca^{2+} in the

presence of millimolar concentrations of Mg^{2+} ($1\ s^{-1}$ for skeletal TnC; Johnson, Charlton & Potter, 1979).

The amount of Ca^{2+} released

A length reduction of about 10% led to rise in aequorin light of about 10–15%, representing an increase in $[Ca^{2+}]$ of 4–6%. It is desirable to determine the size of the change in troponin binding constant which would be required to bring about this change in $[Ca^{2+}]$. Unfortunately we are unable to answer this question mainly because of our inability to explore the changes in light over a range of activating $[Ca^{2+}]$.

This problem is best understood by reference to a very simple model depicted in Fig. 7. This model shows the $[Ca^{2+}]$ and Ca^{2+} bound to troponin (CaT) when various amounts of Ca^{2+} are added to a fixed amount of troponin (70 μM; Fabiato, 1983). In each case the continuous line has a binding constant of K ($= 2 \times 10^6\ M^{-1}$) while the dashed line has a binding constant of $K/2$ ($= 10^6\ M^{-1}$). Thus the difference between the pairs of continuous and dashed lines shows how $[Ca^{2+}]$ and [CaT] would change in a system with a fixed amount of Ca^{2+} if reduction in length led to a 2-fold decrease in the binding constant of troponin for Ca^{2+}. Notice that in a model of this kind, which has no other Ca^{2+} binding, a change in binding constant hardly affects the Ca^{2+} bound to troponin (maximum change 2·6%) whereas the $[Ca^{2+}]$ changes by a factor of 2 at low total Ca^{2+} but is little affected at high total calcium, where troponin becomes saturated with Ca^{2+}. The fractional increase in [Ca] when the binding constant falls is shown as Δ[Ca] (thus a doubling of $[Ca^{2+}]$ appears as a $\Delta[Ca^{2+}]$ of 100%). Note that an increase in $[Ca^{2+}]$ of 4–6%, similar to our experimentally observed result, is obtained when total added Ca^{2+} is between 90 and 100 μmol. The best evidence for a model of this sort would be to show that the fractional increase in [Ca] rises and reaches a limiting value as the total Ca^{2+} added is reduced; as noted earlier technical problems prevented us doing this. The model described so far suggests that the change in K is constant irrespective of the Ca^{2+} added to the system. If, as we suggest, change in length affects K only by virtue of changes in tension brought about initially by changes in myofilament overlap, then changes in the binding constant will only occur when tension is present. This situation is indicated by the line marked Δ[Ca] $\times$ [CaT], since [CaT] determines tension development. In such a model the fractional increase in [Ca] would have a peak at some intermediate level of added Ca^{2+} and decline towards zero on either side of this value. Thus even a simple model of this sort makes it clear that we cannot estimate the change in K from a single estimate of Δ[Ca]. In fact, of course, our experiment is more complex in a variety of ways; in particular we calculate that the presence of Ca^{2+} buffers (EGTA and ATP) in the myoplasm will reduce the observed change in $[Ca^{2+}]$ by a factor of more than 2.

Is it muscle length or tension which determines calcium binding?

These experiments were designed originally to see if the Ca^{2+} affinity of troponin increased with muscle length in a way that could account for the length dependence of myofibrillar Ca^{2+} sensitivity. Although it was length (and sarcomere length) that were altered experimentally, the results suggest that the Ca^{2+} affinity of troponin may depend directly upon cross-bridge attachment rather than upon length itself. The main evidence for this is that, after the restretch of the muscle to L_{max}, the fall of light had a time course that was similar to that of tension and much slower than that of the length change. This association between the light and tension changes continued to be seen when the rate of tension redevelopment was changed by altering the $[Ca^{2+}]$ (Fig. 4) or when the extent of tension redevelopment was varied by changing the initial length (Fig. 6). On this basis, when the muscle is lengthened, the relatively slow re-attachment of cross-bridges to actin (Herzig & Ruegg, 1977;

Brenner & Eisenberg, 1986) produces a slow increase in tension and a slow increase in the Ca^{2+} affinity of TnC; the latter causes $[Ca^{2+}]$ and light to fall. It is known that cross-bridge attachment (in the rigor conformation) increases the Ca^{2+} affinity of TnC (Bremel & Weber, 1972), and in particular the Ca^{2+} affinity of the regulatory Ca^{2+}-specific site(s) of TnC (Wnuk, Schoechlin & Stein, 1984). However, it has not been conclusively shown that cross-bridge attachment in the normal cross-bridge cycle increases the Ca^{2+} affinity of TnC, though there is a body of indirect evidence to support this view (Allen & Kentish, 1985*a*). Our findings are consistent with a role for cross-bridge attachment in influencing the Ca^{2+} affinity of TnC. A similar conclusion was reached by Gordon & Ridgway (1987) in a study on intact barnacle muscle fibres. Although the Ca^{2+} release on muscle shortening can most easily be explained by a decrease in the *number* of attached cross-bridges producing the fall in Ca^{2+} affinity of troponin, we cannot rule out the possibility that it is a change in the conformation, rather than the number, of attached cross-bridges that causes the alteration of troponin's affinity.

Conclusion

The results indicate that the Ca^{2+} affinity of TnC in cardiac myofibrils decreases if the muscle length is reduced; this effect can explain, at least in part, the length dependence of myofibrillar Ca^{2+} sensitivity. The data also provide evidence that the change in Ca^{2+} affinity may depend upon cross-bridge attachment, rather than be related directly to muscle length.

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