Tension-dependent changes of the intracellular Ca^{2+} transients in ferret ventricular muscles

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1. We measured the change in intracellular Ca^{2+} transients, using aequorin, in response to muscle length change during twitch contraction in ferret ventricular muscles.

2. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was transiently increased when the muscle length was quickly shortened to 92% of maximum length (L_{max}) at various times after stimulation (this increase in $[Ca^{2+}]$ is termed extra-Ca^{2+}). The magnitude of extra-Ca^{2+}, measured at different extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$), showed a dependence upon the magnitude of tension reduction and upon $[Ca^{2+}]_i$ immediately before the length change.

3. In the presence of caffeine (5 mM), the difference between the Ca^{2+} transient at L_{max} and at shorter lengths showed a time course similar to the difference between the developed tension at both lengths. A quick release in the caffeine-treated preparation produced the extra-Ca^{2+} with a slower time course compared with that observed in the absence of caffeine. Stretching the muscle from 96% L_{max} to L_{max} produced more active tension and decreased $[Ca^{2+}]_i$.

4. These results indicate that the affinity of troponin-C, a major Ca^{2+} binding protein, which controls contraction, is influenced by developed tension i.e. cross-bridge attachment and detachment.

Contraction of mammalian cardiac muscle is regulated by the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ebashi & Endo, 1968) and the binding of Ca^{2+} to troponin-C is the one of the most important steps in the initiation of contraction (Holroyde, Robertson, Johnson, Solaro & Potter, 1980). If Ca^{2+} is bound to the regulatory site of troponin-C, the cross-bridges attach to thin filaments and contraction is induced. The recent development of the means to measure $[Ca^{2+}]_i$ revealed the relation between $[Ca^{2+}]_i$ and tension during contraction in intact cardiac muscles (Allen & Kurihara, 1980). The tension development of cardiac muscle is significantly influenced by muscle length but the mechanism is not fully understood (Allen & Kentish, 1985). Allen & Kurihara (1982) measured Ca^{2+} transients in mammalian cardiac muscles at different initial muscle lengths before stimulation. They found that the immediate effect of muscle length change alters the decay time of Ca^{2+} transients. They also measured the Ca^{2+} transients when the muscle length was quickly altered during a twitch contraction, and observed a transient increase in the $[Ca^{2+}]_i$ in response to shortening of muscle length. However, stretching the muscle did not produce a change in $[Ca^{2+}]_i$. A transient increase in $[Ca^{2+}]_i$ in response to step length changes is considered to be due to the changes in the affinity of troponin-C for Ca^{2+} which is induced by cross-bridge detachment. Other early reports support this view (Housmans, Lee & Blinks, 1983; Hofmann & Fuchs, 1987*a*; Allen & Kentish, 1988; Kurihara, Saeki, Hongo, Tanaka & Suda, 1990; Saeki, Kurihara, Hongo & Tanaka, 1993). A similar change in Ca^{2+} transient induced by muscle length change is also reported in barnacle muscles (Ridgway & Gordon, 1984; Gordon & Ridgway, 1990). However, the mechanism of the transient change in $[Ca^{2+}]_i$ in response to step length changes in mammalian cardiac muscles has not been fully investigated. In the present study, using aequorin, we measured the alterations of intracellular Ca^{2+} transients in papillary muscles of ferrets when the muscle length was changed under various conditions. The preliminary results have already been presented at the annual meeting of the Japanese Society of Physiology (Kurihara, Komukai & Kawai, 1994*a*) and at the International Conference on Heart Failure (Kurihara, Komukai & Kawai, 1994*b*).

METHODS

Preparations

Ferrets (500–900 g body weight) were anaesthetized with sodium pentobarbitone (80 mg kg^{-1} I.P.) and the hearts were quickly removed. After washing the hearts with normal Tyrode solution, the right ventricle was opened. Thin papillary muscles (0·5–0·9 mm diameter) were dissected and both ends of the preparation were tied with thin silk threads. The diameter of the preparations was 0·65 ± 0·02 mm (mean ± S.E.M., $n = 32$) and the length was 4·3 ± 0·7 mm (mean ± S.E.M.).

Tension recording, length change and stimulation

One end of the preparation was connected to the arm of a tension transducer (BG-10, Kulite, NJ, USA; compliance, 2·5 $\mu m\ g^{-1}$; unloaded resonant frequency, 1 kHz) and the other end was connected to the lever of a motor (JOCX-101A, General Scanning Co. Inc., CA, USA). The motor was used to alter muscle length within 3 ms unless otherwise mentioned. The preparation was mounted in a muscle chamber with a pair of platinum wires placed in parallel with the preparation for electrical stimulation. A parabolic mirror was placed under the preparation to collect the scattered light of aequorin (Allen & Kurihara, 1982). Before the start of the experiment the muscle length was adjusted to L_{max}, the length at which the developed tension reached maximum. Generally, the preparation was stimulated regularly by square pulses with a 5 ms duration at 0·2 Hz, and the strength of the stimulation was 1·5 times threshold.

Solutions

The normal Tyrode solution used for dissecting the preparations and for the injection of aequorin contained (mM): Na^+, 135; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 102; HCO_3^-, 20; HPO_4^{2-}, 1; SO_4^{2-}, 1; acetate, 20; glucose, 10; insulin, 5 units l^{-1}; pH, 7·35 at 30 °C when equilibrated with 5% CO_2–95% O_2. In most experiments, Tyrode solution buffered with Hepes was employed (Hepes–Tyrode solution), which had the following composition (mM): Na^+, 128; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 117; SO_4^{2-}, 1; acetate, 20; Hepes, 5; glucose, 10; insulin, 5 units l^{-1}; pH was adjusted to 7·40 with NaOH at 30 °C. The solution was equilibrated with 100% O_2. When $[Ca^{2+}]_o$ was altered, the osmotic pressure of the solution was not adjusted and $CaCl_2$ was added to or removed from the solution. The temperature of the solution was continuously monitored with a thermocouple and was maintained at 30 ± 0·5 °C.

Aequorin injection and measurement of light signals

Aequorin purchased from Dr J. R. Blinks (Friday Harbor, WA, USA) was dissolved in 150 mM KCl and 5 mM Hepes solution at pH 7·0 with a final aequorin concentration of 50–100 μM. A glass micropipette with a resistance of 30–50 MΩ, measured after filling with aequorin solution, was used for the injection of aequorin. Aequorin was pressure injected into about 150–200 superficial cells of each preparation by monitoring the membrane potential. Aequorin light signals were detected with a photomultiplier (EMI 9789A, Ruislip, UK) which was mounted in a small housing. All data were stored on a tape (NFR-3515W, Sony Magnescale Inc., Tokyo, Japan) and a computer (PC-9801, NEC Corp., Tokyo, Japan) for later analysis. In some experiments, we used a storage oscilloscope (7T07A, NEC San-ei Co., Ltd, Tokyo, Japan) in order to measure the magnitude of tension reduction induced by rapid muscle length changes. The light signals in twitch contraction were recorded through 500 Hz low-pass filters. Sixty-four signals were averaged to improve the signal-to-noise ratio of Ca^{2+} signals.

Aequorin light signals were converted to intracellular Ca^{2+} concentrations using the *in vitro* calibration curve which was measured using a method similar to model B of Allen, Blinks & Prendergast (1977). The constants used in the present study were obtained by rapidly mixing the aequorin which had been pre-equilibrated with 1 mM $MgCl_2$ with solutions having various concentrations of Ca^{2+} at 30 °C. The composition of the solution used for the calibration curve was described in Kurihara & Konishi (1987). Values of the Model B constants (Allen *et al.* 1977) used in the present study were as follows: number of Ca^{2+} binding sites (n), 3·14; equilibrium constants: K_R, $4{\cdot}025 \times 10^6$ and K_{TR}, 114·6.

Drugs

The caffeine used (Sigma) was of analytical grade.

RESULTS

Effects of step length changes on the Ca^{2+} transient and tension

We applied step length changes to the regularly stimulated (frequency, 0·2 Hz) preparation at different times before and after stimulation. Two records, that measured at L_{max} and that when the muscle length was shortened to 92% of L_{max}, were superimposed and the difference between the Ca^{2+} transients (extra-Ca^{2+}) is shown in Fig. 1 (a typical record of 18 experiments). A quick release of the muscle suddenly reduced the tension, which was followed by re-developed tension. In response to the length change, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was transiently increased (extra-Ca^{2+}). We altered the time between the stimulus and the length change. When the time of the length change after the stimulus was delayed, the magnitude of the tension reduction and the magnitude of the extra-Ca^{2+} were increased. However, the Ca^{2+} transient was decayed and the $[Ca^{2+}]_i$ immediately before the length change in the later phase was lower than that in the early phase near the peak. When the length change was applied late after stimulus, the time to peak and the decay time of the extra-Ca^{2+} were shortened. In addition, the time to peak of the re-developed tension was also shortened. When the muscle length was shortened 50 ms before stimulation, the extra-Ca^{2+} was very small and the difference of tension in the rising phase was smaller than that induced by the length change after stimulation (Fig. 1*A*). Thus, the magnitude of the extra-Ca^{2+} was correlated with the magnitude of tension reduction rather than length change. However, we considered that the $[Ca^{2+}]_i$ immediately before the length change was another factor which influences the extra-Ca^{2+}. This will be shown in the following section.

Extra-Ca^{2+} measured at different $[Ca^{2+}]_o$

We measured extra-Ca^{2+} by applying step length changes to the preparation treated with various $[Ca^{2+}]_o$ (Fig. 2; a representative result of 12 experiments). When $[Ca^{2+}]_o$ was increased from 1 to 8 mM, the peaks of Ca^{2+} transients and tension were increased. We altered the muscle length to 92% L_{max} at various times after the stimulus in the solution containing different $[Ca^{2+}]_o$. Then, we measured the relation between the magnitude of extra-Ca^{2+} and of tension reduction in response to the length changes in the solutions with different $[Ca^{2+}]_o$. At each $[Ca^{2+}]_o$, the peak of extra-Ca^{2+} was increased when the magnitude of the tension reduction became larger. However, when the time of the length change after the stimulus was delayed, the $[Ca^{2+}]_i$ immediately before the length change became smaller (due to the decay of Ca^{2+} transient) and the magnitude of tension reduction became larger. Therefore, we considered that two factors are involved in the determination of the magnitude of the extra-Ca^{2+}. Firstly, the magnitude of tension reduction and secondly, $[Ca^{2+}]_i$ immediately before length change. In the following experiments, we altered the muscle length to produce the same magnitude of tension reduction, which shows the dependence of extra-Ca^{2+} on $[Ca^{2+}]_i$ immediately prior to length change.

Extra-Ca^{2+} induced by the same magnitude of tension reduction

We applied the length change to 92% L_{max} to the preparation at 2 mM $[Ca^{2+}]_o$, and measured the magnitude of tension reduction on the storage oscilloscope. We increased $[Ca^{2+}]_o$ and determined the time of length change after the stimulus which produced the same magnitude of tension reduction as in the case of 2 mM $[Ca^{2+}]_o$. With increased $[Ca^{2+}]_o$, the time courses and the peaks of Ca^{2+} transients and tension were changed. At a higher $[Ca^{2+}]_o$,

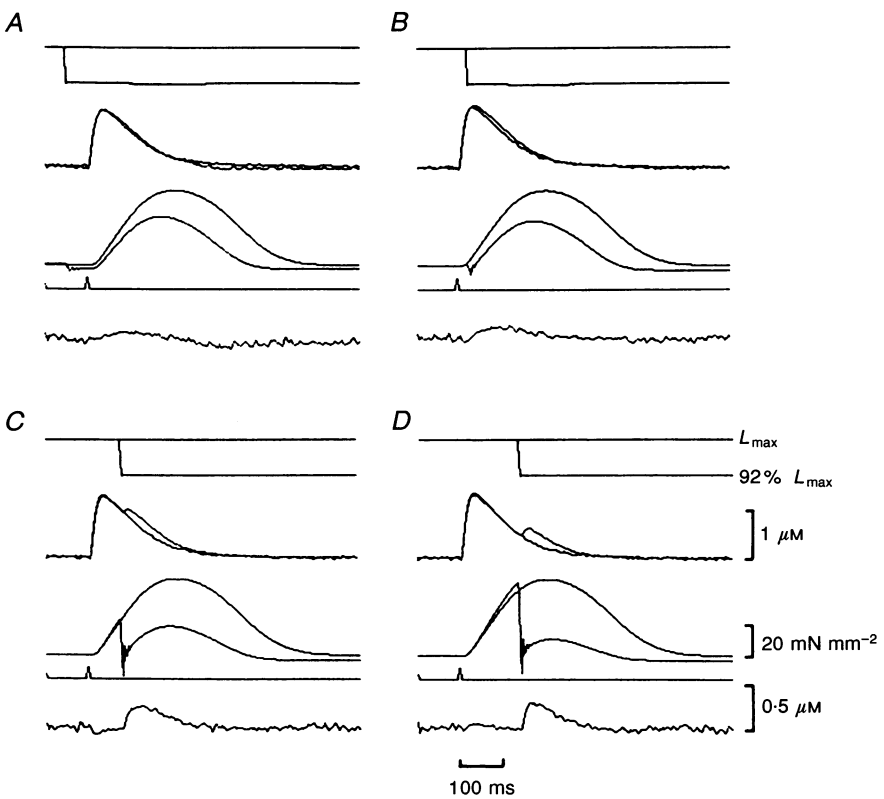

Figure 1. Effects of quick release on intracellular Ca^{2+} transients and tension in twitch contraction

Ca^{2+} transients and tension were measured at L_{max} and when the length change (92% of L_{max}) was applied at various times after (*B*, *C*, and *D*) and before (*A*) stimulation. In each panel, two records (at L_{max} and in the length change) are superimposed. Top trace, muscle length; second trace, $[Ca^{2+}]_i$; third trace, tension; bottom trace, the difference of the Ca^{2+} transients at L_{max} and in the release. In *A*, the muscle length was altered 50 ms before the stimulus. In *B*, *C* and *D*, the muscle length was changed 22, 75 and 138 ms after the stimulus, respectively. Sixty-four signals were averaged to improve the signal-to-noise ratio. The slightly larger tension immediately before release at 138 ms (*D*), compared with that of the control, is within the variation during the collection of sixty-four signals to improve signal-to-noise ratio.

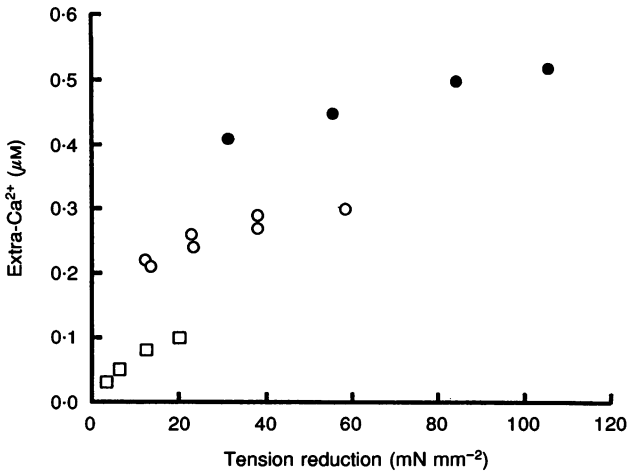

Figure 2. Relation between the magnitude of tension reduction and extra-Ca^{2+} induced by length change at different $[Ca^{2+}]_o$

The tension level and $[Ca^{2+}]_i$ were measured when the length was changed. The magnitude of tension reduction was the tension level immediately before the length change which was measured from resting tension. The tension reduction in the following figures was measured as in Fig. 2. The magnitude of extra-Ca^{2+} was plotted against the magnitude of tension reduction measured at $[Ca^{2+}]_o$ of 1 (□), 2 (○) and 8 (●) mM. At each $[Ca^{2+}]_o$, the magnitude of extra-Ca^{2+} was increased as a larger tension reduction was applied to the preparation. At 2 mM $[Ca^{2+}]_o$, the measurement was repeated twice at the same tension reduction. This figure is a representative result of twelve experiments.

the time of the length change after the stimulus which induced the same magnitude of tension reduction at 2 mM $[Ca^{2+}]_o$ was shortened and the $[Ca^{2+}]_i$ immediately before the length change was higher compared with that at 2 mM $[Ca^{2+}]_o$ (Fig. 3; 12 experiments). The extra-Ca^{2+} was increased in proportion to the increase in $[Ca^{2+}]_i$ immediately before the length change even though the same tension reduction was applied. The analysed typical results are shown in Fig. 4. At the same magnitude of tension reduction (T_1, approximately 25 mN mm^{-2}, Fig. 4*A*), the magnitude of the extra-Ca^{2+} showed a good correlation with $[Ca^{2+}]_i$ immediately before length change.

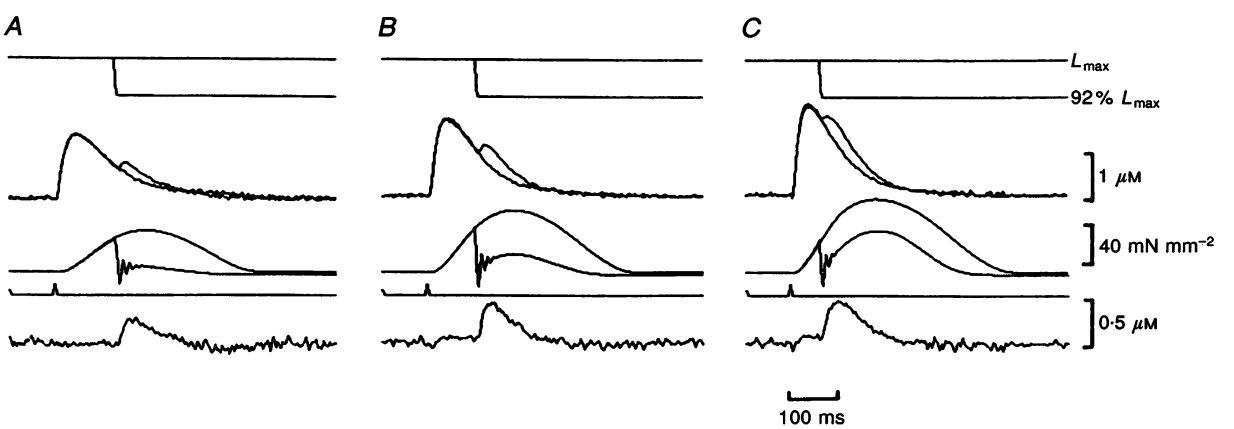

Figure 3. Effects of length change which produces the same magnitude of tension reduction on Ca^{2+} transients at different $[Ca^{2+}]_o$

The time of the length change was determined so as to produce the same magnitude of tension reduction at $[Ca^{2+}]_o$ of 2 (*A*), 4 (*B*) and 8 (*C*) mM. The tension level was measured using a storage oscilloscope and the time of the length change at different $[Ca^{2+}]_o$ to produce the same tension reduction was determined. At different $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ immediately before the length change was different. The slight difference in magnitude of tension reduction was within the variation and did not seriously influence the results. Sixty-four signals were averaged.

A similar dependence of the extra-Ca^{2+} on $[Ca^{2+}]_i$ was observed when the magnitude of tension reduction was increased to T_2 (approximately 50 mN mm^{-2}). Therefore, the peak of the extra-Ca^{2+} was dependent upon both the magnitude of tension reduction and $[Ca^{2+}]_i$ immediately before the length change. In order to eliminate the dependence of extra-Ca^{2+} on $[Ca^{2+}]_i$, the extra-Ca^{2+} was normalized to the $[Ca^{2+}]_i$ immediately before the length change and the normalized extra-Ca^{2+} was plotted against the magnitude of tension reduction (Fig. 4*B*). The normalized extra-Ca^{2+} was increased in proportion to the magnitude of the tension reduction.

Ca^{2+} transients in the caffeine-treated preparations of different initial muscle lengths

We considered that the extra-Ca^{2+} was removed by Ca^{2+} handling mechanisms, particularly by sarcoplasmic reticulum (SR) and that prolongation of the time course of both Ca^{2+} transient and tension by caffeine enhanced the extra-Ca^{2+}. Thus, we treated the preparations with 5 mM caffeine which is known to prolong the time courses of both Ca^{2+} transients and tension (Allen & Kurihara, 1980). In the presence of 5 mM caffeine, Ca^{2+} transients showed a plateau which was followed by a slower decay phase and the time course of tension was prolonged. Therefore, we could examine the effects of length change on tension and Ca^{2+} transients in the plateau of the Ca^{2+} transients (relatively constant $[Ca^{2+}]_i$). The slower time courses of Ca^{2+} transients and tension might be attributable to the slower Ca^{2+} uptake by the SR and an increase in the Ca^{2+} sensitivity of the contractile elements (Fig. 5) (Weber & Herz, 1968; Wendt & Stephenson, 1983). We set the initial muscle length at 96% of L_{max} and measured Ca^{2+} transients and tension. The measured records were compared with those at L_{max} (3 experiments). At the shorter muscle length, the developed tension was lower than that at L_{max} but the peak of Ca^{2+} transients was slightly larger than that at L_{max}. The difference in the Ca^{2+} transients at both lengths became obvious when the difference of the developed tension was clearly observed. In the early phase of contraction in which the peak of the developed tension was similar at both lengths, no clear difference in Ca^{2+} transients was observed. A similar tension-dependent change in the difference in Ca^{2+} transients was more clearly observed when the initial muscle length was set at an even shorter length (92% L_{max}; 3 experiments). Therefore, the difference in Ca^{2+} transients at two different initial muscle lengths is related to the difference of the developed tension rather than length.

Effects of release and stretch on the caffeine-treated preparations

We applied mechanical perturbations to the preparations treated with 5 mM caffeine and 8 mM Ca^{2+} which prolonged and increased both Ca^{2+} transients and tension. When the

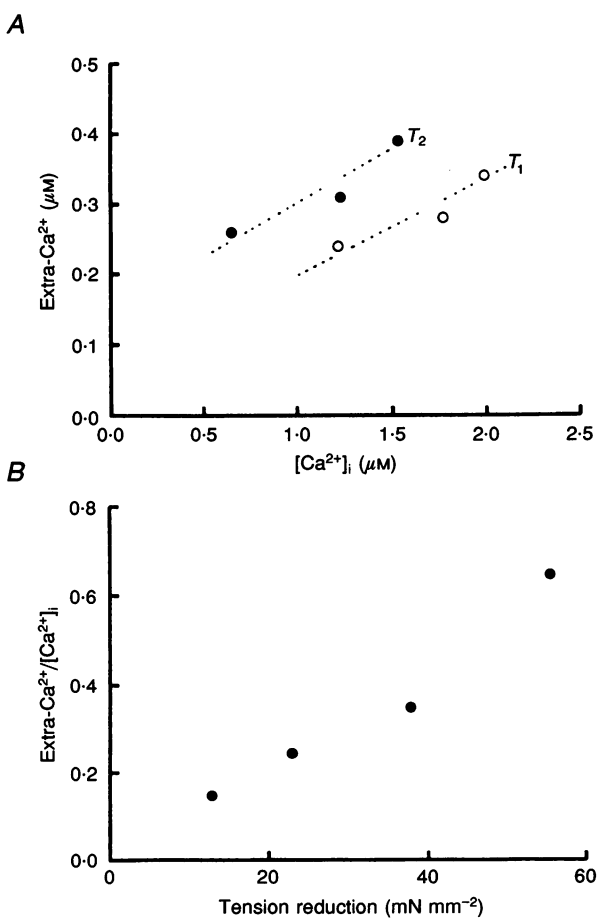

Figure 4. Dependence of extra-Ca^{2+} on $[Ca^{2+}]_i$ and tension reduction

Relation between the magnitude of extra-Ca^{2+} and $[Ca^{2+}]_i$ immediately before the length change which produced the same magnitude of tension reduction (T_1 and T_2; *A*). Length change was to 92% L_{max}. The procedure was the same as that used in Fig. 3 and tension reduction with two different magnitudes (T_1 and T_2) were applied. T_1 and T_2 were approximately 25 and 50 mN mm^{-2}, respectively. In *B*, extra-Ca^{2+} was normalized to $[Ca^{2+}]_i$ immediately before the length change and plotted against the magnitude of tension reduction.

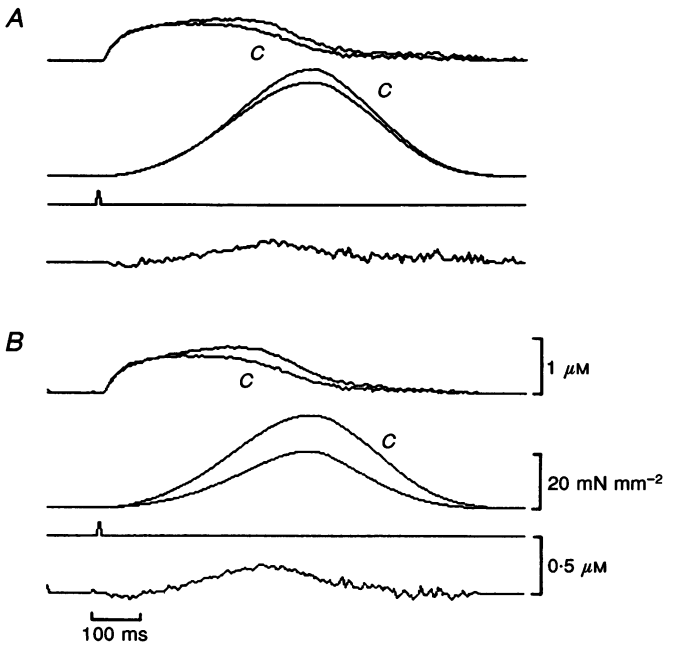

Figure 5. Effects of initial muscle length on Ca^{2+} transients and tension in the preparation treated with caffeine (5 mM)

In the presence of caffeine, the time course of Ca^{2+} transients and tension was slow and a plateau was recognized in the Ca^{2+} transients. The Ca^{2+} transients measured at L_{max} (labelled *C*) and shorter lengths (96% L_{max} in *A* and 92% L_{max} in *B*) were compared. In the bottom trace in each panel, the difference in Ca^{2+} transients measured at L_{max} and shorter lengths is shown. The appearance of the extra-Ca^{2+} approximately corresponded to the difference of tension at both lengths, which is clearly observed in *B*; no extra-Ca^{2+} was observed at the initial phase of the Ca^{2+} transients even though the muscle length was altered before stimulation. Sixty-four signals were averaged.

muscle length was shortened to 92% L_{max} 144 ms after stimulus, which corresponded to the plateau of the Ca^{2+} transient, the tension was suddenly reduced and then re-developed. In response to the release, $[Ca^{2+}]_i$ was transiently increased as in the absence of caffeine (Fig. 6*A*). The time course of the extra-Ca^{2+} in the presence of caffeine was much longer than that in the absence of caffeine (6 experiments). The initial muscle length was then set at 96% of L_{max} and the Ca^{2+} transient was measured as a control. The preparation was quickly stretched from 96% L_{max} to L_{max} 144 ms after the stimulus. Stretching the muscle induced a short-lived transient increase in tension which was followed by an increased active tension. In accordance with the stretch, $[Ca^{2+}]_i$ was decreased, which was shown as a negative deflection of the extra-Ca^{2+} (Fig. 6*B*; 4 experiments).

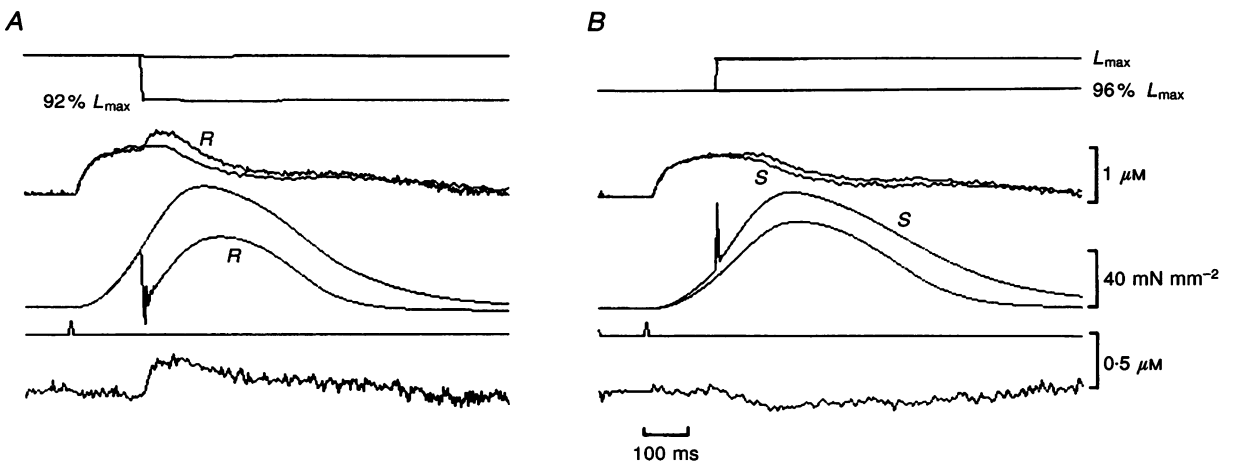

Figure 6. Effects of length change on Ca^{2+} transients and tension in caffeine (5 mM)-treated preparations

$[Ca^{2+}]_o$ was 8 mM to potentiate the Ca^{2+} transients. In *A*, two records measured at L_{max} and when the muscle length was shortened to 92% L_{max} (*R*) are superimposed. Extra-Ca^{2+} is shown in the bottom trace. In *B*, the initial muscle length was set at 96% L_{max} and Ca^{2+} transients and tension were measured. Then, the preparation was quickly stretched to L_{max} at the same time as the release occurred in *A*. In the stretch, a transient and short-lived transient tension was recognized. Stretching the preparation (*S*) produced more tension following the short-lived transient tension (*S*) and decreased Ca^{2+} transients (*S*). In the bottom trace the difference between Ca^{2+} transients at 96% L_{max} and in the stretch is shown. The slightly larger tension immediately before stretching the preparation (*B*) is within the variation of tension with the averaging of sixty-four signals.

DISCUSSION

The origin of extra-Ca^{2+}

The present experiments showed that mechanical perturbations applied during twitch contraction alter $[Ca^{2+}]_i$. Releasing the muscle during twitch contraction produced a transient increase in $[Ca^{2+}]_i$ and stretching the muscle treated with caffeine decreased $[Ca^{2+}]_i$. As shown in Fig. 4, the magnitude of the extra-Ca^{2+} was dependent upon both the magnitude of the tension reduction induced by a quick release and $[Ca^{2+}]_i$ immediately before the length change. If $[Ca^{2+}]_i$ is increased following stimulation, Ca^{2+} binds to the regulatory site of troponin-C which causes cross-bridge attachment and thus produces tension (Holroyde *et al.* 1980; Pan & Solaro, 1987). The relation between $[Ca^{2+}]_i$ and the amount of the Ca^{2+}-binding form of troponin-C (Tn–Ca complex) is not linear. However, if $[Ca^{2+}]_i$ is higher, more Tn–Ca complex is formed (Pan & Solaro, 1987). As shown in Fig. 4, the dependence of extra-Ca^{2+} on $[Ca^{2+}]_i$ supports the hypothesis that the origin of extra-Ca^{2+} is Ca^{2+} bound to troponin-C as suggested previously (Allen & Kurihara, 1982; Allen & Kentish, 1985; Hofmann & Fuchs, 1987*a*, *b*; Kurihara *et al.* 1990; Saeki *et al.* 1993).

The measurement of the extra-Ca^{2+} in the relaxation phase would provide a means to further test the hypothesis that the origin of the extra-Ca^{2+} is Ca^{2+} bound to troponin-C. In barnacle muscles, the extra-Ca^{2+} was measured during relaxation (Ridgway & Gordon, 1984). However, the Ca^{2+} transient substantially decreases after the peak of tension in ferret papillary muscle. Therefore, the amount of Ca^{2+} bound to troponin-C is also decreased and the small extra-Ca^{2+} in the later phase of contraction cannot be accurately measured. In barnacle muscles, the time courses of Ca^{2+} transients and tension are closely related to each other and sufficient Ca^{2+} remains on the Ca^{2+} binding sites of troponin-C even in the later phase of Ca^{2+} transients. Thus, quick release can still produce extra-Ca^{2+} in the later phase of Ca^{2+} transients. Therefore, the time lag between the Ca^{2+} transient and tension characterizes the profile of the extra-Ca^{2+}. We must also consider the possibility that extra-Ca^{2+} is due to Ca^{2+} influx from the extracellular space or Ca^{2+} release from the intracellular Ca^{2+} store (sarcoplasmic reticulum, SR). However, it is unlikely because the change in the Ca^{2+} concentration in the bathing solution of skinned preparations treated with Triton X-100, which corresponded to the extra-Ca^{2+}, can also be observed in response to length changes (Allen & Kentish, 1985). We cannot completely exclude the possibility that the extra-Ca^{2+} comes from intracellular Ca^{2+} binding sites other than troponin-C (myosin, actin and calmodulin). However, these are not considered to be the origin of Ca^{2+} released by mechanical perturbations (see Discussion in Allen & Kentish, 1985).

The dependence of extra-Ca^{2+} on the changes in tension

If length change is an essential factor for producing the extra-Ca^{2+}, the peak of extra-Ca^{2+} should correlate with the change in the muscle length. However, the extra-Ca^{2+} produced by the muscle length change which was applied immediately before stimulus was smaller than that induced by the muscle length change after stimulus (Fig. 1*A*). Similarly, the quick release immediately after the peak of Ca^{2+} transients which corresponded to a small tension development produced a small extra-Ca^{2+} (Fig. 1*B*). In the caffeine-treated preparation, the difference in $[Ca^{2+}]_i$ was not observed until the difference in the developed tension was clearly observed although the muscle length had changed (Fig. 5). These results further support the idea that extra-Ca^{2+} is closely related to the change in tension rather than the change in muscle length as suggested previously (Allen & Kurihara, 1982; Housmans, Lee & Blinks, 1983; Lab, Allen & Orchard, 1984; Allen & Kentish, 1988; Kurihara *et al.* 1990; Saeki *et al.* 1993). However, no significant changes in Ca^{2+} transients were observed when the muscle was stretched during a twitch contraction under physiological conditions (Allen & Kurihara, 1982; Kurihara *et al.* 1990). In addition, stretching the muscle from a shorter length to the original length before the release during tetanic contraction does not produce a similar change in Ca^{2+} signals which is observed in the release (Saeki *et al.* 1993). Therefore, in mammalian cardiac muscles, the changes in $[Ca^{2+}]_i$ in response to release and stretch are different and in fact asymmetrical. However, in the present study, $[Ca^{2+}]_i$ was decreased by stretching the caffeine-treated muscle from a shorter length (96 % L_{max}) to the L_{max}. The decrease in the Ca^{2+} transient corresponded to the increase in the developed tension. However, no significant change in Ca^{2+} transient was observed in accordance with the short-lived transient tension change which appeared immediately after the stretch (Fig. 6*B*). Similarly, stretching a tetanized preparation from a shorter length to L_{max} also produces a transient and short-lived tension change but the Ca^{2+} signal is not influenced (Saeki *et al.* 1993). However, the $[Ca^{2+}]_i$ is decreased in response to the stretch which corresponds to the delayed tension development (Saeki *et al.* 1993). In addition, the time course of the decay of the Ca^{2+} signal induced by the length change is similar to that of the delayed tension development. These results suggest that $[Ca^{2+}]_i$ is decreased if more active tension is produced by stretching the muscle as in the case of barnacle single fibres (Gordon & Ridgway, 1990). However, if the cross-bridges are forcibly detached by stretching the muscle, which might occur in a short-lived transient tension, no significant changes in the Ca^{2+} signals are induced (Fig. 6*B* and Saeki *et al.* 1993). Therefore, the development of active tension (not passive tension) which is induced by cross-bridge attachment is an

essential factor for the decrease in $[Ca^{2+}]_i$. We considered that if more tension is produced by the attachment of the cross-bridges, this increases the affinity of troponin-C for Ca^{2+}, leading to a decrease in $[Ca^{2+}]_i$. Thus, the attachment of cross-bridges is essential for the increase in the affinity of troponin-C for Ca^{2+}. On the other hand, the detachment of the cross-bridges decreases the affinity of troponin-C for Ca^{2+}. The more effective role of the cycling cross-bridge in changing the affinity of the regulatory site of troponin also supports our view (Guth & Potter, 1987; Hofmann & Fuchs, 1987*a*, *b*). However, Gulati, Sonnenblick & Babu (1991) consider length change to be a factor involved in the effect of stretching the muscle. In caffeine-treated preparations, the time course of tension was prolonged, which might be due to the slower Ca^{2+} removal from the myoplasm and the increase in the Ca^{2+} sensitivity of the contractile elements (Allen & Kurihara, 1980; Wendt & Stephenson, 1983). The mechanism of the caffeine-induced increase in the Ca^{2+} sensitivity of the contractile elements is controversial due to the following findings: (1) there is no change in the Ca^{2+} binding to isolated troponin-C (Palmer & Kentish, 1994); (2) there is an increase in the Ca^{2+} binding to troponin-C which is complexed with other subunits (troponin-I and troponin-T) (Liao & Gwathmey, 1994), and (3) there is no substantial change in the Ca^{2+} binding to troponin-C in skinned preparations (Powers & Solaro, 1994). If caffeine increases the Ca^{2+} binding to troponin-C, then more Tn–Ca complex (the origin of extra-Ca^{2+}) is formed and thus the influence of the cross-bridge attachment and detachment is enhanced (Fig. 6). However, even though the amount of Ca^{2+} bound to troponin-C is not increased by caffeine (Palmer & Kentish, 1994; Powers & Solaro, 1994), the slower removal of the extra-Ca^{2+} in the caffeine treatment could explain the change in $[Ca^{2+}]_i$ accompanying the slower time course in response to release and stretch. Thus, the rate of Ca^{2+} removal is one of the factors related to the change in $[Ca^{2+}]_i$ induced by mechanical perturbations. In addition, if caffeine prolongs the interaction of the cross-bridges with thin filaments, this might enhance the $[Ca^{2+}]_i$ change in response to release and stretch. Although the mechanism of the effects of caffeine is not clear, potentiation of tension by caffeine and the substantial change in active tension is an important trigger for the change in $[Ca^{2+}]_i$.

Quantitative consideration of extra-Ca^{2+}

The present study showed that the extra-Ca^{2+} induced by releasing muscle during a twitch in the Hepes–Tyrode solution was 0·1–0·5 μM. The extra-Ca^{2+} did not exceed 1 μM as far as we measured under various conditions. Recently, Backx & Ter Keurs (1993) also reported a small change in $[Ca^{2+}]_i$ measured with fura-2 in response to a quick release during a twitch. As discussed above, Ca^{2+} removal mechanisms, particularly that of SR, are responsible for the decay of the extra-Ca^{2+}. Therefore, the Ca^{2+} removal mechanisms and the Ca^{2+} binding to the binding sites including troponin-C (re-binding of the extra-Ca^{2+} to troponin-C) profoundly influence the estimation of the tension-dependent change in the affinity of troponin-C for Ca^{2+}. Thus, the comparison of the results in intact preparations and in skinned preparations is not straightforward (Pan & Solaro, 1987). Further studies regarding the quantification of the tension-dependent change in the affinity of troponin-C for Ca^{2+} are necessary.

Allen, D. G., Blinks, J. R. & Prendergast, F. G. (1977). Aequorin luminescence: relation of light emission to calcium concentration – a calcium-independent component. *Science* **195**, 996–998.

Allen, D. G. & Kentish, J. C. (1985). The cellular basis of the length–tension relation in cardiac muscle. *Journal of Molecular and Cellular Cardiology* **17**, 821–840.

Allen, D. G. & Kentish, J. C. (1988). Calcium concentration in the myoplasm of skinned ferret ventricular muscle following changes in muscle length. *Journal of Physiology* **407**, 489–503.

Allen, D. G. & Kurihara, S. (1980). Calcium transients in mammalian ventricular muscle. *European Heart Journal* **1**, 1–15.

Allen, D. G. & Kurihara, S. (1982). The effects of muscle length on intracellular calcium transients in mammalian cardiac muscle. *Journal of Physiology* **327**, 79–94.

Backx, P. H. & Ter Keurs, H. E. D. J. (1993). Fluorescent properties of rat cardiac trabeculae microinjected with fura-2 salt. *American Journal of Physiology* **264**, H1098–1110.

Ebashi, S. & Endo, M. (1968). Calcium ion and muscle contraction. *Progress in Biophysics and Molecular Biology* **18**, 123–183.

Gordon, A. M. & Ridgway, E. B. (1990). Stretch of active muscle during the declining phase of the calcium transient produces biphasic change in calcium binding to the activating sites. *Journal of General Physiology* **96**, 1013–1035.

Gulati, J., Sonnenblick, E. & Babu, A. (1991). The role of troponin C in the length dependence of Ca^{2+}-sensitive force of mammalian skeletal and cardiac muscles. *Journal of Physiology* **441**, 305–324.

Guth, K. & Potter, J. D. (1987). Effects of rigor and cycling cross-bridges on the structure of troponin C and on the Ca^{2+} affinity of the Ca^{2+}-specific regulatory sites in skinned rabbit psoas fibers. *Journal of Biological Chemistry* **262**, 13627–13635.

Hofmann, P. A. & Fuchs, F. (1987*a*). Effect of length and cross-bridge attachment on Ca^{2+} binding to cardiac troponin C. *American Journal of Physiology* **253**, C90–96.

Hofmann, P. A. & Fuchs, F. (1987*b*). Evidence for a force-dependent component of calcium binding to cardiac troponin-C. *American Journal of Physiology* **253**, C541–546.

Holroyde, M. J., Robertson, S. P., Johnson, J. D., Solaro, R. J. & Potter, J. D. (1980). The calcium and magnesium binding sites on cardiac troponin and their role in the regulation of myofibrillar adenosine triphosphate. *Journal of Biological Chemistry* **255**, 11688–11693.

Housmans, P. R., Lee, N. K. M. & Blinks, J. R. (1983). Active shortening retards the decline of the intracellular calcium transient in mammalian heart muscle. *Science* **221**, 159–161.

Kurihara, S., Komukai, K. & Kawai, M. (1994*a*). Effects of mechanical perturbation on intracellular Ca^{2+} transients and tension in ferret ventricular muscles. *Japanese Journal of Physiology* **44**, suppl. 1, S82.

KURIHARA, S., KOMUKAI, K. & KAWAI, M. (1994*b*). Intracellular Ca transients in response to step length changes in aequorin-injected ferret papillary muscles. *Canadian Journal of Cardiology* **10**, suppl. A, 48A.

KURIHARA, S. & KONISHI, M. (1987). Effects of β-adrenoceptor stimulation on intracellular Ca transients and tension in rat ventricular muscle. *Pflügers Archiv* **409**, 427–437.

KURIHARA, S., SAEKI, Y., HONGO, K., TANAKA, E. & SUDA, N. (1990). Effects of length change on intracellular Ca^{2+} transients in ferret ventricular muscle treated with 2,3-butanedione monoxime (BDM). *Japanese Journal of Physiology* **40**, 915–920.

LAB, M. J., ALLEN, D. G. & ORCHARD, C. H. (1984). The effects of shortening on myoplasmic calcium concentration and on the action potential in mammalian ventricular muscle. *Circulation Research* **55**, 825–829.

LIAO, R. & GWATHMEY, J. K. (1994). Effects of MCI-154 and caffeine on Ca^{2+}-regulated interactions between troponin subunits from bovine heart. *Journal of Pharmacology and Experimental Therapeutics* **270**, 831–839.

PALMER, S. & KENTISH, J. C. (1994). The role of troponin-C in modulating the Ca^{2+} sensitivity of mammalian skinned cardiac and skeletal muscle fibres. *Journal of Physiology* **480**, 45–60.

PAN, B.-S. & SOLARO, R. J. (1987). Calcium-binding properties of troponin C in detergent-skinned heart muscle fibers. *Journal of Biological Chemistry* **262**, 7839–7849.

POWERS, F. M. & SOLARO, R. J. (1994). Caffeine affects dog cardiac myofilament activation independent of Ca^{2+} binding to troponin C (TnC). *Journal of Molecular and Cellular Cardiology* **26**, CLVII.

RIDGWAY, E. B. & GORDON, A. L. (1984). Muscle calcium transient: Effect of post-stimulus length changes in single fibers. *Journal of General Physiology* **83**, 75–103.

SAEKI, Y., KURIHARA, S., HONGO, K. & TANAKA, E. (1993). Alterations in intracellular calcium and tension of activated ferret papillary muscle in response to step length changes. *Journal of Physiology* **463**, 291–306.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and effect of caffeine on reticulum. *Journal of General Physiology* **52**, 750–759.

WENDT, I. R. & STEPHENSON, D. G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibers of the rat. *Pflügers Archiv* **398**, 210–216.

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