Cross-Bridge-Dependent Change of the Ca^{2+} Sensitivity During Relaxation in Aequorin-Injected Tetanized Ferret Papillary Muscles

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Background The aim of the present study was to indicate the cross-bridge-dependent change in the Ca^{2+} affinity of troponin-C (TnC) during relaxation in an intact preparation, because the intracellular mechanism of relaxation is not fully understood, although several methods of evaluating global diastolic function have been reported.

Methods and Results The aequorin method was used with intact ferret papillary muscles and a tetanic contraction was induced by a repetitive electrical stimulation in the presence of ryanodine. The extra- Ca^{2+} , the transient increase in the intracellular Ca^{2+} concentration in response to a rapid reduction in muscle length, which reflects the change in the Ca^{2+} affinity of TnC because of cross-bridge detachment, was measured, and the cross-bridge-dependent change in the Ca^{2+} affinity of TnC was estimated by observing the change in the slope of the extra- Ca^{2+} -tension relation. The extra- Ca^{2+} -tension relation measured during relaxation became steeper than that during contraction in all cases. The extra- Ca^{2+} -tension relation became steeper in the presence of 20 mmol/L caffeine during contraction in all cases.

Conclusion During relaxation, the downstream-dependent change in the Ca^{2+} affinity of TnC was enhanced, compared with that during contraction, because of a decrease in the number of attached cross-bridges. (Circ J 2006; 70: 913–918)

Key Words: Ca^{2+} sensitivity; Caffeine; Cross-bridge; Diastolic dysfunction; Relaxation; Troponin-C

D iastolic dysfunction was recently highlighted as the underlying mechanism of congestive heart failure! so it is important to evaluate the normal diastolic function of the heart and several methods have been developed^{2–4}

The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) regulates contraction and is one of the key factors in cardiac muscle relaxation^{5,6}. A decrease in $[\text{Ca}^{2+}]_i$ dissociates Ca^{2+} from troponin-C (TnC), which precedes relaxation, and therefore, the Ca^{2+} uptake rate of the sarcoplasmic reticulum (SR) is one of the determinants of the rate of relaxation. However, factors other than Ca^{2+} uptake rate of SR are suggested to be involved in the determination of the rate of relaxation; for example, the Ca^{2+} sensitivity of the contractile elements and the cross-bridge detachment^{5,6}. Recent studies indicated that the cross-bridge detachment decreases the Ca^{2+} sensitivity and these 2 factors are inter-related by a feedback mechanism from the attached cross-bridges to TnC⁷ (downstream mechanism: see Terminology⁸ in Methods section below and Fig 1). Therefore, it is difficult to differentiate these 2 mechanisms and in order to clarify the intracellular Ca^{2+} regulatory mechanism of relaxation, the property of downstream mechanism during relaxation has to be evaluated.

We recently reported that the cross-bridge (downstream)-dependent change in the Ca^{2+} sensitivity of the myofila-

ments can be estimated by measuring the transient change in $[\text{Ca}^{2+}]_i$ induced by shortening muscle length in intact papillary muscles injected with aequorin^{9–15}. However, in twitch, $[\text{Ca}^{2+}]_i$ and TnC are not equilibrated and the Ca^{2+} transient returns to almost resting level at the peak of twitch tension^{5,6}. Thus, it is difficult to measure the $[\text{Ca}^{2+}]_i$ change in relaxation of twitch.

In the present study, we aimed to measure the transient change in $[\text{Ca}^{2+}]_i$ induced by muscle length reduction during tetanic contraction in which intracellular Ca^{2+} and TnC are equilibrated^{11–13}. During tetanic contraction, the influence of the SR on relaxation can be excluded because tetanic contraction is induced in the presence of ryanodine. Thus, we could examine how relaxation is influenced by the cross-bridge-dependent change in the Ca^{2+} sensitivity.

Methods

Preparation

Our experimental procedure was approved by the Guidance for Animal Experiments at The Jikei University School of Medicine. Adult male ferrets were anesthetized with pentobarbital sodium (100 mg/kg ip) and the heart was quickly removed. After washing away the blood by retrograde perfusion with normal Tyrode's solution through the aorta, the thin papillary muscles were dissected from the right ventricle in a bath continuously perfused with normal Tyrode's solution at $30 \pm 0.5^\circ\text{C}$. Both ends of the preparation were tied with silk threads. One end of the preparation was connected to the lever of a motor (JCCX-101A, General Scanning Co Inc, Watertown, MA, USA), which was used to alter the length of the preparation, and the other end was connected to the arm of a tension transducer (BG-10, Kulite,

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Fig 1. A model of the facilitation of the cardiac contraction-relaxation cycle by downstream-dependent change of the Ca^{2+} sensitivity. In contraction (Contraction: Upper side), $[\text{Ca}^{2+}]_i$ was increased by the Ca^{2+} induced- Ca^{2+} release by the sarcoplasmic reticulum (SR). The increase in the number of attached cross-bridge further enhances the Ca^{2+} sensitivity through the feedback mechanism,⁷ which potentiates contraction (indicated by solid arrows). On the other hand, in relaxation (Relaxation: Lower side), the number of attached cross-bridge is reduced, which further reduces the Ca^{2+} sensitivity through the feedback mechanism,⁷ facilitating relaxation (indicated by dot arrows). Therefore, the downstream-dependent change of the Ca^{2+} affinity of troponin-C (TnC) functions as the cellular mechanism of the Frank-Starling's law of the heart facilitating the continuous contraction-relaxation cycle (see details in Discussion). $[\text{Ca}^{2+}]_i$ returns to the resting level, preceding relaxation, by Ca^{2+} re-uptake by SR and membranous Ca^{2+} removal mechanisms. The definitions of the 3 steps in the intracellular regulatory mechanism of cardiac muscle contraction (ie, upstream, central, and downstream mechanisms) are described in the Terminology subsection of the Methods.

Semiconductor Products, Inc, Leonia, NJ, USA; compliance, $2.5 \mu\text{m/g}$; unloaded resonant frequency, 1 kHz). The preparation was mounted horizontally in an experimental chamber with a pair of platinum black electrodes placed parallel to the preparation for electrical stimulation. The preparation was stimulated with a rectangular pulse at 1.2-fold threshold with 5 ms duration. The stimulation frequency was 0.2 Hz unless otherwise stated. Before starting the experiment, the preparation was gradually stretched from the slack length to L_{max} , the length at which developed tension reached maximum. The muscle length of the preparation was $3.63 \pm 0.69 \text{ mm}$ (mean $\pm$ standard deviation), and the diameter of the preparation was $0.726 \pm 0.202 \text{ mm}$ ($n=8$).

Measurement of $[\text{Ca}^{2+}]_i$ With Aequorin

The injection of aequorin and the measurement of aequorin's light signal were essentially similar to the previous reports.^{12–15} The light signal of aequorin was converted to $[\text{Ca}^{2+}]_i$ using the following equation, which was obtained by an in vitro calibration curve:

$$I/I_{\text{max}} = [(1 + K_R[\text{Ca}^{2+}]) / (1 + K_{\text{TR}} + K_R[\text{Ca}^{2+}])]^n$$

where I indicates aequorin light intensity, and I_{max} indicates peak light intensity at saturating Ca^{2+} (20 mmol/L). In the experiments, I_{max} was calculated from the total light, which was obtained by mixing all intracellular aequorin with Ca^{2+} in the solution using Triton-X. The constants used for the conversion of the aequorin light signal to $[\text{Ca}^{2+}]_i$ were the same as previously reported from our laboratory: n , 3.14; K_R , 4025000; K_{TR} , 114.6.¹⁶

Solutions

Normal Tyrode's solution was used for both dissection and injection of aequorin (composition in mmol/L : 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; pH, 7.35 at 30°C when equilibrated with 5% CO_2 with 95% O_2).

In the experiments, Tyrode's solution was buffered with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid) and had the following composition (mmol/L): Na^+ , 128; K^+ , 5; Ca^{2+} , 2; Mg^{2+} , 1; Cl^- , 117; SO_4^{2-} , 1; HEPES, 5; acetate, 20; glucose, 10; insulin, 5 units/L; pH was adjusted to 7.40 with NaOH at 30°C . The solution was equilibrated with 100% O_2 . When the Ca^{2+} concentration in the solution ($[\text{Ca}^{2+}]_o$) was altered, CaCl_2 was either added to, or removed from, the solution without adjusting the osmotic pressure of the solution. The temperature of the solution was continuously monitored with a thermocouple and maintained at $30 \pm 0.5^\circ\text{C}$.

Terminology

Blinks and Endo divided the intracellular regulatory mechanism of cardiac muscle contraction into 3 steps (upstream, central, and downstream),⁸ which is shown briefly in Fig 1. The upstream step is mainly related to the change in $[\text{Ca}^{2+}]_i$ which is regulated by the influx of Ca^{2+} , Ca^{2+} release from the SR, Ca^{2+} uptake by SR, and Ca^{2+} extrusion through the cell membrane. The central step is Ca^{2+} binding to TnC (Ca^{2+} sensitivity in narrow definition). The downstream step is the attachment of cross-bridges after Ca^{2+} binding to TnC. The attached active cross-bridge is known to influ-

ence the central mechanism (Ca²⁺ sensitivity) through the feedback mechanism (downstream-dependent mechanism or cross-bridge-dependent mechanism).⁷

Experimental Protocols

Induction of a Tetanic Contraction A tetanic contraction was induced by an electrical repetitive stimulation in the presence of 5 μ mol/L ryanodine as previously reported.^{11–13,15} About 4 s later, developed tension and [Ca²⁺]_i become nearly steady-state, and stimulation was continued for 10 s (contraction phase). After cessation of stimulation, developed tension and [Ca²⁺]_i gradually decreased and returned to the resting level (relaxation phase).

Measurement of the Extra-Ca²⁺ in a Tetanic Contraction To assess the cross-bridge-dependent change in Ca²⁺ sensitivity, we measured the [Ca²⁺]_i change (extra-Ca²⁺) during a tetanic contraction by quickly shortening muscle length (quick length change method). The measurement of the extra-Ca²⁺ in a tetanic contraction was essentially similar to that reported previously.^{11–13} Briefly, during a tetanic contraction, 6 s after the onset of stimulation, the muscle length of the preparation was quickly shortened from L_{max} within 4 ms using the electromagnetic motor and 2 s later (8 s after the onset of stimulation), the preparation was quickly stretched to the initial muscle length (L_{max}). In response to the quick shortening of the muscle length, a transient increase in [Ca²⁺]_i (extra-Ca²⁺) occurs.^{9–15} We measured the magnitude of the extra-Ca²⁺ by changing the magnitude of tension reduction from L_{max} to 96%, 92%, 88%, 84%, or 80% L_{max}. A further 2 s after the cessation of stimulation (12 s after the onset of contraction), during relaxation phase, muscle length was quickly reduced as described above, and the extra-Ca²⁺ was observed. Eight to 16 signals were averaged to improve the signal-to-noise ratio.

The origin of the extra-Ca²⁺ and the rationale to use this method for the evaluation of the extra-Ca²⁺-tension relation are described in our previous reports^{9,10,13,14} and also explained in the Discussion below. In brief, the extra-Ca²⁺ magnitude is a function of the magnitude of tension reduction and [Ca²⁺]_i immediately before the length change.¹⁰ To assess the dependence of the Ca²⁺ affinity of TnC on tension reduction, the magnitude of the extra-Ca²⁺ normalized to [Ca²⁺]_i immediately before length change (Extra-Ca²⁺/[Ca²⁺]_i) was plotted at least 2 points against the magnitude of tension reduction [Tension reduction] in each preparation, and the linear line was drawn. In order to draw the linear relation, 2 points as the approximately minimum and maximum magnitude of tension reduction changes were needed to be measured. Therefore, by reducing the length from L_{max} to 96% L_{max} or 92% L_{max} (in case the extra-Ca²⁺ was too small to detect by reducing to 96% L_{max}), the minimum point was plotted. By reducing to 80% L_{max} or 84% L_{max} (in case the magnitude of tension reduction was almost the same as for 80% L_{max}), the maximal point was plotted. When more than 3 points were obtained, the extra-Ca²⁺-tension relation was delineated as the regression line of those points. In some experiments, another point as the middle magnitude of tension reduction change was measured, in order to delineate the regression line with several points. The absolute value of the slope of the extra-Ca²⁺-tension relation delineated by 3 points did not quantitatively differ from that by 2 points.^{10,13,14}

The slope of the line (the extra-Ca²⁺-tension relation) represents the dependence of the change in the Ca²⁺ affinity for TnC on the magnitude of tension reduction.^{10,12–15} When

the slope of the extra-Ca²⁺-tension relation becomes shallow, more active cross-bridges are formed, which is due to the downstream-dependent increase in Ca²⁺ sensitivity.^{13,14} The opposite is also true. Because there was discrepancy in the developed tension and [Ca²⁺]_i among the preparations, the absolute values of the slope of the line were different. Therefore, the change in the slope of the extra-Ca²⁺-tension relation was compared qualitatively in each experiment.

Extra-Ca²⁺-Tension Relation During Contraction and Relaxation The extra-Ca²⁺ was measured at 15 mmol/L [Ca²⁺]_o as in the previous studies.^{11–13} The extra-Ca²⁺-tension relations during contraction and relaxation were investigated by comparing the change in the slope of the extra-Ca²⁺-tension relation.

Effects of 20 mmol/L Caffeine on the Extra-Ca²⁺-Tension Relation During Contraction In order to further investigate the difference between contraction and relaxation, the effect of a high concentration (20 mmol/L) of caffeine on the downstream-dependent change of Ca²⁺ sensitivity was investigated. This was because a high concentration of caffeine reduces the number of attached cross-bridge, as reflected in the significant reduction in maximal activated tension^{17,18} by increasing the cross-bridge kinetics,¹⁷ which simulates the state of cross-bridge kinetics during relaxation. First, we tried to observe the effect of a high concentration of caffeine on the extra-Ca²⁺ during relaxation. However, possibly because of the increased cross-bridge kinetics,¹⁷ the relaxation time became too fast in the presence of 20 mmol/L caffeine. Therefore, with the reduction in the maximal activated tension in the presence of caffeine,^{17,18} the extra-Ca²⁺ could not be measured during relaxation. Thus, we tested the effect of 20 mmol/L caffeine on the extra-Ca²⁺ during contraction as a simulation of the state of cross-bridge kinetics during relaxation.

The extra-Ca²⁺ was measured in a contraction phase in the absence (Control) and presence of 20 mmol/L caffeine (Caffeine) in 15 mmol/L [Ca²⁺]_o, and the slopes of the extra-Ca²⁺-tension relation were compared.

Chemicals

Aequorin was purchased from Dr J. R. Blinks (Friday Harbor, WA, USA). Ryanodine was purchased from AgriSystem (Wind Gap, PA, USA), and 1 mmol/L stock solution was prepared by dissolving it in warm double-distilled water, then stored at 0°C. Caffeine was purchased from Sigma Chemical Co Ltd (MO, USA) and dissolved directly in the phosphate-free Tyrode's solution before use.

Results

Extra-Ca²⁺-Tension Relation During Contraction and Relaxation

Fig 2 shows a representative record of the developed tension, Ca²⁺ transient, expressed as [Ca²⁺]_i, which was calculated from the light signal using the constants (see Methods), and the extra-Ca²⁺ during contraction and relaxation. The quick shortening of the muscle length produced a transient decrease in tension and a transient increase in [Ca²⁺]_i. The extra-Ca²⁺ during contraction and relaxation are also shown.

Fig 3 shows the relation between extra-Ca²⁺ and the magnitude of tension reduction during contraction and relaxation (representative data from 4 experiments). The extra-Ca²⁺-tension relation measured during relaxation (Relaxation) ($Y=0.0118X-0.0347$) became steeper than

Fig2. Extra- Ca^{2+} during contraction and relaxation in tetanized myocardium. Representative records measuring the extra- Ca^{2+} during contraction (Left) and relaxation (Right) (Upper trace; developed tension, Middle trace; Ca^{2+} transients ($[\text{Ca}^{2+}]_i$) converted from aequorin light, Lower trace; extra- Ca^{2+}) (representative record from 4 experiments). Downward arrow in the upper trace shows the shortening of muscle length from L_{max} to $84\%\text{L}_{\text{max}}$ and upward arrow shows the stretch of muscle from $84\%\text{L}_{\text{max}}$ to L_{max} . Small humps in the Ca^{2+} transients are the extra- Ca^{2+} (). Two records are superimposed in the Upper and Middle panels (records of the developed tension and Ca^{2+} transient). The smooth records without humps and notches are the control. The records with transient changes in the Ca^{2+} transients and tension were obtained when the muscle length was quickly shortened from L_{max} to $84\%\text{L}_{\text{max}}$.

Fig3. Extra- Ca^{2+} -tension relations during contraction and relaxation. The extra- Ca^{2+} was normalized by dividing Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by immediately before length change (the normalized extra- Ca^{2+} ($[\text{Extra-}\text{Ca}^{2+}]/[\text{Ca}^{2+}]_i$)). In all 4 cases, Extra- $\text{Ca}^{2+}/[\text{Ca}^{2+}]_i$ was increased with the magnitude of tension reduction (Tension reduction). The line measured during relaxation (Relaxation) (: $Y=0.0118X-0.0347$) became steeper compared with during contraction (Contraction) (: $Y=0.00154X-0.0510$) (representative case from 4 experiments).

that during contraction (Contraction) ($Y=0.00154X-0.0510$). This qualitative increase in the slope of the line during relaxation was consistently observed in all 4 experiments.

Fig4. Effect of 20 mmol/L caffeine on the extra- Ca^{2+} -tension relation during contraction. The extra- Ca^{2+} -tension relation became steeper in the presence of 20 mmol/L caffeine (Caffeine) (: $Y=0.0681X-0.895$) compared with in the absence of caffeine (Control) (: $Y=0.00977X+0.177$) (representative case from 4 experiments). This was consistent in all 4 experiments.

Effects of 20 mmol/L Caffeine on the Extra- Ca^{2+} -Tension Relation

Fig4 shows a representative result of the effects of 20 mmol/L caffeine on the extra- Ca^{2+} -tension relation during a contraction phase. The reason for using a high concentration of caffeine in the experiments and the effects of a high concentration of caffeine (the increase in the cross-bridge kinetics¹⁷) are described detail in Methods above.

Caffeine (Caffeine) made the extra- Ca^{2+} -tension relation

($Y=0.0681X-0.895$) steeper compared to the data obtained in the absence of caffeine (Control) ($Y=0.00977X+0.177$). This tendency of an increase in the slope of the line in the presence of caffeine was consistently observed in all 4 experiments.

Discussion

The present study directly indicated the downstream-dependent change of Ca²⁺ sensitivity during relaxation, the rate limitation of relaxation¹⁷ and the intracellular mechanism of the Frank-Starling's law of the heart (as described below and shown in Fig 1) in aequorin-injected tetanized intact myocardium (Fig 3). This is the first report to indicate the opposite state of downstream mechanism during contraction and relaxation in intact myocardium. Therefore, if this method was applied to the myocardium of heart failure, the alteration in the downstream mechanism could be investigated, which would contribute to clarification of the intracellular mechanism of diastolic dysfunction in heart failure.

Evaluation of the quantitative change in the slope of the extra-Ca²⁺-tension relation has been previously reported^{10,13,14} and the difference between contraction and relaxation in the slope of the line can be explained as follows. The extra-Ca²⁺ is a function of the magnitude of the tension reduction and [Ca²⁺]_i immediately before the change in length.¹⁰ In order to evaluate the dependence of the Ca²⁺ affinity for TnC on tension, the magnitude of the extra-Ca²⁺ was normalized to [Ca²⁺]_i immediately before the length change (Extra-Ca²⁺/[Ca²⁺]_i) and the normalized extra-Ca²⁺ was plotted against the tension reduction in each prepared muscle¹⁰ (Tension reduction) (Figs 3,4). The positive regression in the relationship between Extra-Ca²⁺/[Ca²⁺]_i and Tension reduction was consistent in both twitch and tetanic contractions.¹³ Thus, the slope of the regression line (the extra-Ca²⁺-tension relation) represents the dependence of the change in the Ca²⁺ affinity for TnC (Ca²⁺ sensitivity) on the magnitude of tension reduction. If the slope of the extra-Ca²⁺-tension relation becomes shallow, the downstream-dependent mechanism is enhanced through the recruitment of cross-bridge attachments caused by enhanced feedback from the cross-bridges to TnC.^{13,14} Thus, a steeper extra-Ca²⁺-tension relation during relaxation indicates that more Ca²⁺ can be easily released from the TnC·Ca²⁺ complex because of the enhancement of the downstream mechanism as a result of the decrease in the number of active cross-bridges (Fig 3). The difference in the state of cross-bridges between contraction and relaxation is modeled in Fig 1. The increase in the slope of the extra-Ca²⁺-tension relation in the presence of 20 mmol/L caffeine, which reduces the number of attached cross-bridge,^{17,18} supports this interpretation (Fig 4). In a former report, similar effects of 2,3-butanedione monoxime, an inhibitor of cross-bridges, and shortening of the initial muscle length of the preparation, thus reducing the number of attached cross-bridges, on the extra-Ca²⁺-tension relation in a tetanus preparation were reported;¹³ and further support our interpretation.

Cross-bridge-dependent change of the Ca²⁺ affinity of TnC (Ca²⁺ sensitivity) is considered to be the cellular mechanism of the Frank-Starling's law of the heart,^{5,12,15,19} although the difference of the mechanism during contraction and relaxation was not fully understood. The present study indicates the opposite state of cross-bridge-dependent

change of the Ca²⁺ affinity for TnC between during contraction and relaxation (Figs 1,3,4). The time courses of [Ca²⁺]_i and contraction were regulated by the downstream mechanism.⁹ Thus, the cross-bridge-dependent change of the Ca²⁺ affinity for TnC functions as the cellular mechanism of the Frank-Starling's law of the heart facilitating the continuous contraction-relaxation cycle as explained in Fig 1. On the other hand, the Ca²⁺ uptake rate of the SR modified the time courses of [Ca²⁺]_i and contraction, as indicated in the pathophysiological conditions of diabetes¹² (slowed Ca²⁺ uptake by SR) and hyperthyroidism¹⁵ (accelerated Ca²⁺ uptake by SR). In addition, a recent study showed that alteration of SERCA2a did not influence the cardiac performance in humans with failing heart.²⁰ Thus, further studies focusing on the downstream mechanism as a therapeutic target of heart failure are necessary.

Conclusion

The downstream-dependent change in the Ca²⁺ sensitivity during relaxation is enhanced compared with during contraction, because of a decrease in the number of cross-bridges.

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