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Microscopic heat pulses induce contraction of cardiomyocytes without calcium transients

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ABSTRACT

It was recently demonstrated that laser irradiation can control the beating of cardiomyocytes and hearts, however, the precise mechanism remains to be clarified. Among the effects induced by laser irradiation on biological tissues, temperature change is one possible effect which can alter physiological functions. Therefore, we investigated the mechanism by which heat pulses, produced by infra-red laser light under an optical microscope, induce contractions of cardiomyocytes. Here we show that microscopic heat pulses induce contraction of rat adult cardiomyocytes. The temperature increase, ΔT, required for inducing contraction of cardiomyocytes was dependent upon the ambient temperature; that is, ΔT at physiological temperature was lower than that at room temperature. Ca^{2+} transients, which are usually coupled to contraction, were not detected. We confirmed that the contractions of skinned cardiomyocytes were induced by the heat pulses even in free Ca^{2+} solution. This heat pulse-induced Ca^{2+}-decoupled contraction technique has the potential to stimulate heart and skeletal muscles in a manner different from the conventional electrical stimulations.

1. Introduction

Optical methods have been developed as a novel non-invasive means to control living cells and animals [1]. Non-contact methods are particularly effective for the stimulation of moving tissues such as heart muscles without interrupting their mechanical functions. The optogenetic approach can precisely induce contraction of heart muscle *in vitro* and *in vivo*, whereas cardiomyocytes must be genetically modified to express light-activated cation channels [2]. On the other hand, intact cardiomyocytes and heart can be optically manipulated by laser irradiations without modification of gene expression [3–5]. The mechanism of contraction induction, however, is not yet clarified. This is because laser irradiation can cause complex reactions, such as photochemical and photothermal effects on biological tissues [6]. Among these effects, it is known that local temperature increases can control various functions of living cells. For example, irradiation of living cells via infrared (IR) laser mediates gene induction [7]. Nerve cells are optically activated by local temperature changes induced by the laser irradiation [8]. Also, we have demonstrated that a Ca^{2+} burst is induced in HeLa cells at body temperature by a heat pulse with amplitudes of temperature changes as small as 0.2 °C [9]. The mechanism is thought to be similar to that of rapid cooling contracture (RCC) [10,11]. Rapid cooling stimulations (typically from room temperature to 4 °C) cause Ca^{2+} release from sarcoplasmic reticulum (SR) mainly through the ryanodine receptor, which triggers Ca^{2+}-induced Ca^{2+} release (CICR) and the calcium transients trigger contraction. In the case of HeLa cells, IP_3 receptors rather than ryanodine receptors are responsible for CICR. Here we examined the effects of microscopic heat pulses on the contraction and Ca^{2+} dynamics in single rat adult cardiomyocytes.

2. Materials and methods

2.1. Preparation of cardiomyocytes

Ventricular cardiomyocytes were prepared from adult rats according to the previously reported method [12–14]. The isolated cardiomyocytes were placed on a glass bottom dish (Asahi Glass Co., Tokyo, Japan) containing the extracellular solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 10 mM HEPES, 2 mM $CaCl_2$, 5 mM D-glucose, pH 7.4 with NaOH) and incubated at 37 °C with 5% CO_2 overnight. For the measurements using

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skinned cardiomyocytes, the cells in a sample tube containing the extracellular solution were cooled on ice during the skinning procedure (within 60 min). All experimental procedures conformed to the "Guidelines for Proper Conduct of Animal Experiments" approved by the Science Council of Japan, and were performed according to the "Guidelines on Animal Experimentation" of The Jikei University School of Medicine and the "Regulations for Animal Experimentation" at Waseda University.

2.2. Optical setup

The fluorescence microscopy was basically the same as that previously described [9], with an objective lens (PlanApo N 60×/1.45 Oil, Olympus, Tokyo, Japan). Excitation filters for microthermometers [15] (BP360–370) and fluo-4 (BP470–490) were switched by using a filter wheel (Lambda 10-3, Sutter Instrument Co., Novato, CA, USA). A dichroic mirror DM505 and an emission filter BA515IF were mounted. Short-pass filters, FF01-945 (Semrock, Inc., Rochester, NY, USA) and SIX870 (Asahi Spectra Co., Tokyo, Japan), were placed in front of the electron multiplying charge coupled device (EM-CCD) camera (iXon EM+ 897, Andor Technology, Belfast, UK). The temperature of the extracellular solution was adjusted by a thermostatically controlled incubator on the sample stage (INUG2-ONICS, Tokai Hit, Shizuoka, Japan), and measured with a digital thermometer (ASF-250T, AS ONE, Osaka, Japan). The solution was directly heated by focusing the infra-red (IR) laser beam (λ = 1455 nm, KPS-STD-BT-RFL-1455-02-CO, Keopsys, Lannion, France) under the microscope. The laser power at the top of the objective lens was measured with a thermal disk sensor and a power meter (LM-3 and FieldMaster, Coherent, Inc., Santa Clara, CA, USA). The ON/OFF of heating was regulated by a shutter system (SSH-C4B, Sigma Koki, Tokyo, Japan) placed in the light path of the IR laser beam and the period of the heat pulse was regulated by a free auto-clicker software. The changes in microscopic temperature, ΔT, were measured with a microthermometer as previously reported [15]. The microthermometer was manipulated by a motorized micromanipulator (EMM-3SV, Narishige, Tokyo, Japan).

2.3. Electrical stimulation

Cardiomyocytes in the extracellular solution were stimulated with the electronic stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Two electrodes generated the voltage gradient of approximately 15 V/cm (the distance between the two electrodes was 3–3.5 cm and the applied voltage was 50 V) for 5 ms. Cells were stimulated at 36 ± 0.5 °C.

2.4. Inhibition of actomyosin interaction

Blebbistatin (25 mM) (Toronto Research Chemicals Inc., North York, Ontario, Canada) in dimethyl sulfoxide (DMSO) (Sigma–Aldrich Co., St. Louis, MO, USA) was diluted to 25 μM in the extracellular solution. The cardiomyocytes were incubated in the extracellular solution containing 25 μM blebbistatin for at least 15 min under the microscope before microheating experiments at 36 ± 0.5 °C.

2.5. Fluo-4 loading for Ca^{2+} imaging

Cardiomyocytes were incubated in the extracellular solution containing 2 μM fluo-4, AM (Invitrogen, Carlsbad, CA, USA) for 30 min at 25 °C. The cells were washed with the extracellular solution, placed under the microscope and observed at 36 ± 0.5 °C.

2.6. Preparation of skinned cells

Cardiomyocytes in a sample tube were washed twice with the relaxing solution [5.8 mM adenosine triphosphate (ATP), 6.9 mM $MgCl_2$, 10 mM EGTA, 15 mM phosphocreatine disodium, 45 mM K-propionate, 40 mM BES, 1 mM DTT, pH 7.0 with KOH] and incubated in the relaxing solution containing 0.3% Triton X-100 (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 60 min at room temperature. The skinned cells were washed twice with the relaxing solution and placed on the glass bottom dish containing the relaxing solution under the microscope. Cells were observed at 36 ± 0.5 °C.

2.7. Image analysis

The cross-sectional area of cardiomyocytes, which was constantly changing, on the x–y plane was manually traced with ImageJ (National Institutes of Health). From the area before heating (A_0) and during heating (A_h), the degree of shortening (%) was calculated as $100 \times (A_0 - A_h)/A_0$. In order to assess the measurement error, we measured the area of cardiomyocytes for the same image twice (A_1 and A_2), and calculated the error as $100 \times (A_1 - A_2)/A_1$. Then, by considering the distribution of the errors as a Gaussian distribution (Fig. S1, N = 101), we determined the measurement error as the standard deviation (SD) × 1.96 = ±0.62 × 1.96 = ± 1.21% where 95% of the error is included. Fluorescence intensities of microthermometers and fluo-4 were also measured with ImageJ. The area of the region of interest for measuring the fluorescence intensity of fluo4, which was chosen around the center of cardiomyocytes, was 424 ± 198 μm^2 (average ± SD).

3. Results

3.1. Contraction of cardiomyocytes induced by microscopic heat pulse

Heat pulse was generated by IR-laser focused in the extracellular solution near cardiomyocytes (Fig. 1A). 1455 nm IR laser light is absorbed by water, so that the focused spot becomes a heat source and the concentric temperature gradient is created around the spot (Fig. 1B and C). The period of heating (Fig. 1B) and the degree of temperature increase (Fig. 1C), ΔT, of heat pulses could be controlled by the illumination period and the laser power, respectively. Here we found that adult cardiomyocytes incubated at 36 °C showed contraction during heating (Fig. 2A and Movie-1). The contraction continued during heating for 0.5 s and the relaxation occurred immediately after turning off the laser. The duration of heat-induced contraction was longer than that of the physiological contraction induced by electrical stimulation (Fig. 2B and Movie-1). The cardiomyocyte oscillated when heat pulses were applied repeatedly (by turning on and off the laser for 200 ms each at 2.5 Hz; Fig. 2C and Movie-2). The cross-section of cardiomyocytes during heating on the x–y plane were arcuately curved, whereas the contraction induced by electrical stimulations occurred along the long axis of cardiomyocytes (Fig. 2D). This difference suggests that the side of cardiomyocytes closer to the heat source contracted stronger than the farther side due to the temperature gradient as shown in Fig. 1C. The degree of shortening, a measure of the strength of contraction, was defined as the ratio of the cross-sectional area of cardiomyocytes during activation to that before activation (Fig. 2E). When the laser-induced maximum temperature change at the centroid of cardiomyocytes was approximately 1, 3.5, 5, and 6.5 °C, the contraction probabilities, defined as the percentage of cells with the degree of shortening larger than the measurement error (1.21%), were 10 (2/20), 52 (13/25), 95 (40/42) and 100% (17/17), respectively. The amplitude of contraction

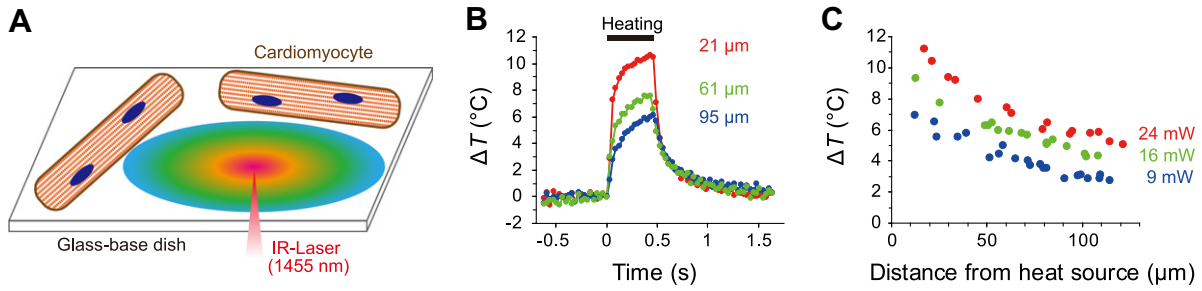

Fig. 1. Microheating and temperature imaging system. (A) A schematic illustration of the microheating system. The temperature in the extracellular solution was directly increased by focusing an IR-laser beam (1455 nm) under an optical microscope. (B) Time courses of temperature changes, ΔT, at various places 21 (red), 61 (green), and 95 μm (blue) distant from the center of the heat source. The black bar indicates the period of heating with the IR-laser (24 mW). (C) Temperature distribution around the heat source obtained under several different laser powers. Laser powers indicated were measured at the top of the objective lens. The ambient temperature was 36 °C.

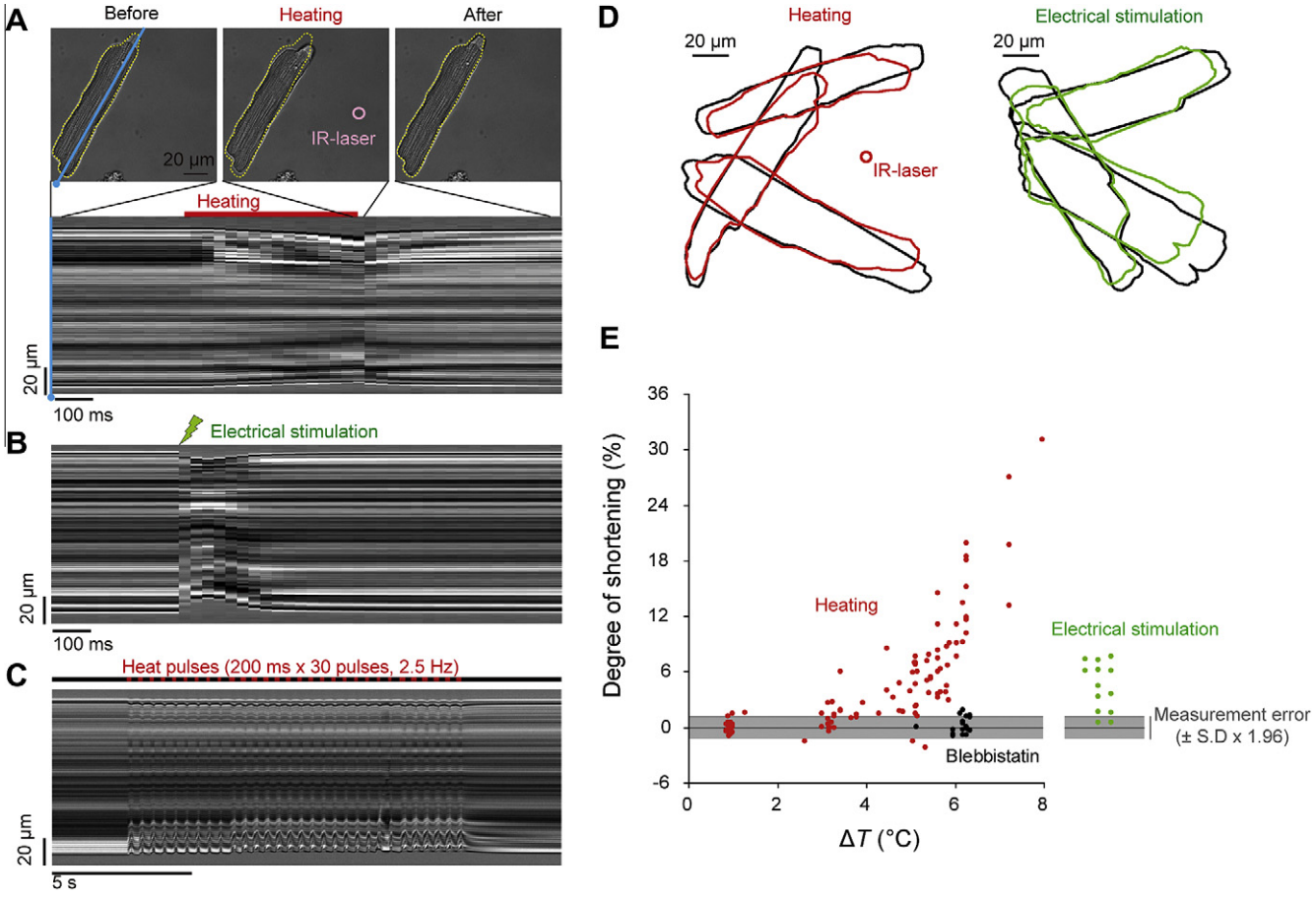

Fig. 2. Contraction of cardiomyocytes induced by heat pulses. (A) Top figures are bright-field images of a cardiomyocyte before, during, and after heating for 0.5 s. The pink circle indicates the position of the heat source. Yellow dashed lines indicate outlines of the cell before heating. The bottom figure is a kymograph of the blue line of the upper-left figure. The red bar indicates the period of heating. (B) A typical kymograph of the contraction induced by the electrical stimulation (5 ms, 15 V/cm). (C) A kymograph of 30 repetitive contractions induced by 30 consecutive heat pulses (approximately 200 ms each, 2.5 Hz). Red bars indicate the period during which heat pulses were applied (red). (D) The outlines of six cardiomyocytes before (black) and during contraction induced by heat pulses for 0.5 s (red) or electrical stimulation (green). The red circle indicates the heat source. (E) The relationship between the average temperature changes, ΔT, and the shortening level of non-treated (red, $N = 104$) or blebbistatin-treated (black, $N = 18$) cardiomyocytes. Green dots indicate the degree of shortening induced by electrical stimulation ($N = 13$). Gray bars show the region within the measurement error (±1.2%). All experiments were done at 36 ± 0.5 °C.

induced by electrical stimulations (15 V/cm for 5 ms) was 4.4 ± 0.3% (average ± SD, $N = 18$). As the degree of temperature change increased, the degree of shortening became larger than that induced by electrical stimulation (Fig. 2E). Blebbistatin, an inhibitor of the actin–myosin interaction in cardiac muscles [16], significantly ($p < 10^{-14}$) blocked the contraction induced by the heat pulse irrespective of the degree of temperature change (Fig. 2E). This result indicates that the contraction is attributable to heat-activated actin–myosin interaction, but not to the passive mechanical bending, for example, due to the convection flow.

3.2. Contraction of cardiomyocytes occurs by a smaller temperature jump in the approach to the physiological temperature

The value of ΔT required for contraction at room temperature (25 °C) was higher than that at physiological temperature (36 °C)

(Fig. 3A and B). At the room temperature, ΔT of 11.5 °C was required for contraction of 63% of cardiomyocytes ($N = 27$), whereas at 36 °C ΔT of approximately 4 °C was sufficient for the contraction of a similar percentage of cells (Fig. 3B). When we focused on the absolute temperature during heating (ambient temperature + ΔT) at two different ambient temperatures (Fig. 3C and D), we found that cardiomyocytes hardly contracted when heated from 36 to 37 °C, whereas more than half (63%) contracted when heated from 26.5 to 36.5 °C. This result may be attributable to the fact that cardiomyocytes had already pre-contracted at 36 °C. However, the cross-sectional area of cardiomyocytes prepared from the same rats at 25 and 36 °C were 3056 ± 595 μm^2 (average ± SD, $N = 66$) and 3146 ± 562 μm^2 ($N = 65$), respectively (Fig. S2). This indicates that no significant difference in the volume of cardiomyocytes existed between the two preparation conditions ($p > 0.05$), implying that the possibility is very low that the cardiomyocytes prepared at 36 °C pre-contracted before the heat pulse. These results suggest that a rapid temperature increase, ΔT, but not an absolute temperature, T, is important for the contraction.

3.3. Heat pulse-induced contraction occurs without Ca^{2+} transients

Different from the case observed in HeLa cells [9], Ca^{2+} transients were not observed coupled with the heat pulse (Fig. 4A), although electrical stimulation did induce Ca^{2+} transients as expected (Fig. 4B). The maximum changes in fluorescence intensity of fluo-4 loaded into cardiomyocytes, which were induced during heating or by electrical stimulation, were, respectively, 0.2 ± 0.6 and 2.0 ± 1.4 (average ± SD) times of those before activations (Fig. 4C). In these experiments, the ambient temperature was 36 °C and the average temperature increase, ΔT, was 5.3 ± 0.4 °C, which caused contraction as strong as that induced by electrical stimulation (Fig. 2E). The results suggest that the heat pulse induces contraction of cardiomyocytes without Ca^{2+} transients.

To confirm this conclusion, we examined the effects of heat pulse on skinned cardiomyocytes, so that we could examine the effects of heat pulse under controlled solvent conditions. As a result, we found that the heat pulse-induced contraction occurred in Ca^{2+}-free solution (Fig. 4D and Movie-3). Two-thirds (4/6) of skinned cardiomyocytes contracted when heated from 36 to 42–43 °C, although the degree of shortening was smaller than that of intact cardiomyocytes.

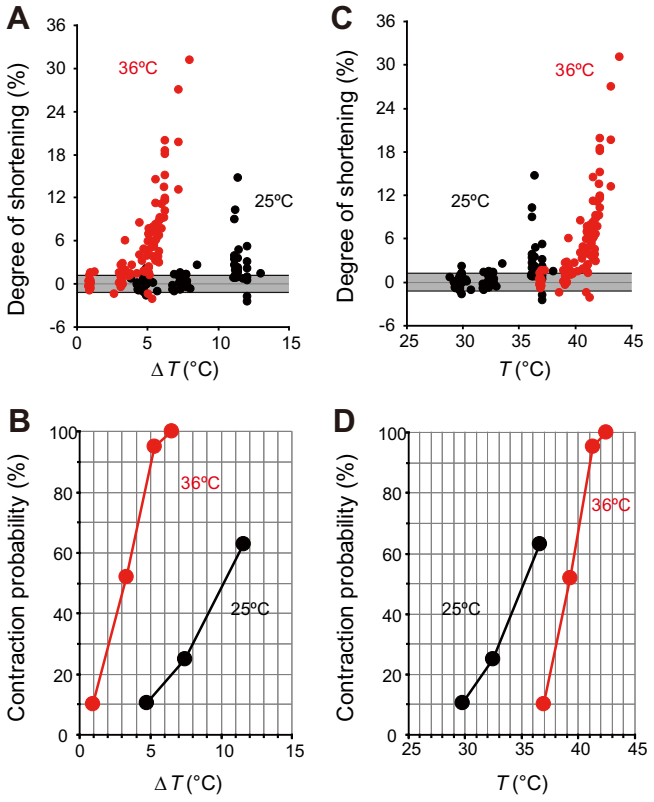

Fig. 3. Effects of ambient temperature on the heat pulse-induced contraction. (A) The relationship between temperature change, ΔT, induced by heat pulse for 0.5 s and the degree of shortening. (B) The relationship between temperature change, ΔT, and the contraction probability. (C and D) The relationship between temperature during heating (T = ambient temperature + ΔT) and the degree of shortening (C) or the contraction probability (D). Ambient temperatures were 36 ± 0.5 °C (red, $N = 104$) or 25 ± 0.5 °C (black, $N = 66$). Gray bars in (A and C) indicate the region within the measurement error.

4. Discussion

We found that microscopic heat pulses repetitively induce contractions in cardiomyocytes. The temperature increase necessary for inducing the contraction of cardiomyocytes at physiological temperature (36 °C) was lower than that at room temperature (25 °C). The tendency is similar to the previous study in HeLa cells [9]. In HeLa cells, the overshoot of Ca^{2+} release occurs above the critical value of a temperature change, and this critical temperature change decreases from 1.5 to 0.2 °C on increasing the experimental (ambient) temperature from 22 to 37 °C. Both results of cardiomyocytes and HeLa cells are in agreement with the idea that living cells have highly thermosensitive systems at physiological temperature, although the manner of response to the heat pulse of cardiomyocytes was different from that of HeLa cells, i.e., the absence of Ca^{2+} burst and contraction independent of Ca^{2+} dynamics.

The present results demonstrated that heat pulses induce contractions of cardiomyocytes without Ca^{2+} transients. Physiological contractions induced by electrical stimulation follow several steps from the stimulation to the contraction, called excitation–contraction coupling [17]. First, the depolarization induced by electrical stimulation activates voltage-dependent Ca^{2+} channels, which causes Ca^{2+} entry through the channels. This Ca^{2+} influx triggers the release of Ca^{2+} from SR through ryanodine receptors, known as Ca^{2+} induced Ca^{2+} release. The Ca^{2+} binding to troponin-C triggers the movement and/or conformational change of tropomyosin on actin filament (F-actin), which activates the interaction between F-actin and myosin to initiate the muscle contraction. Our results strongly suggest that microheating stimulation bypasses the steps related to Ca^{2+} signaling and directly activates actomyosin interactions (Fig. 4E).

How do the cardiomyocytes contract without Ca^{2+} transients? One of the most probable mechanisms is a partial dissociation of tropomyosin from F-actin induced by heating. It has been reported that tropomyosin reversibly dissociates from F-actin at 35–40 °C in the absence of troponin [18] and at 45 °C in the presence of troponin without Ca^{2+} [19,20]. In these studies, the dissociation of tropomyosin from F-actin was detected by measuring the degree of flow birefringence of mixtures containing these proteins. Although the local movements within the tropomyosin molecule on F-actin could not be detected in these measurements, the weakening of interaction between tropomyosin and actin is expected to occur below the dissociation temperature. Moreover, it is well established that the binding of Ca^{2+} to troponin is not sufficient for full activation of the muscle, rather cooperative activation as a result of cross-bridge formation is required for the full activation [21,22]. Single-molecule experiments have revealed that the number of cross-bridges increases with temperature [23,24]. Therefore, the positive feedback by the cross-bridge formation to the activation of the thin filaments could also play an important part in the

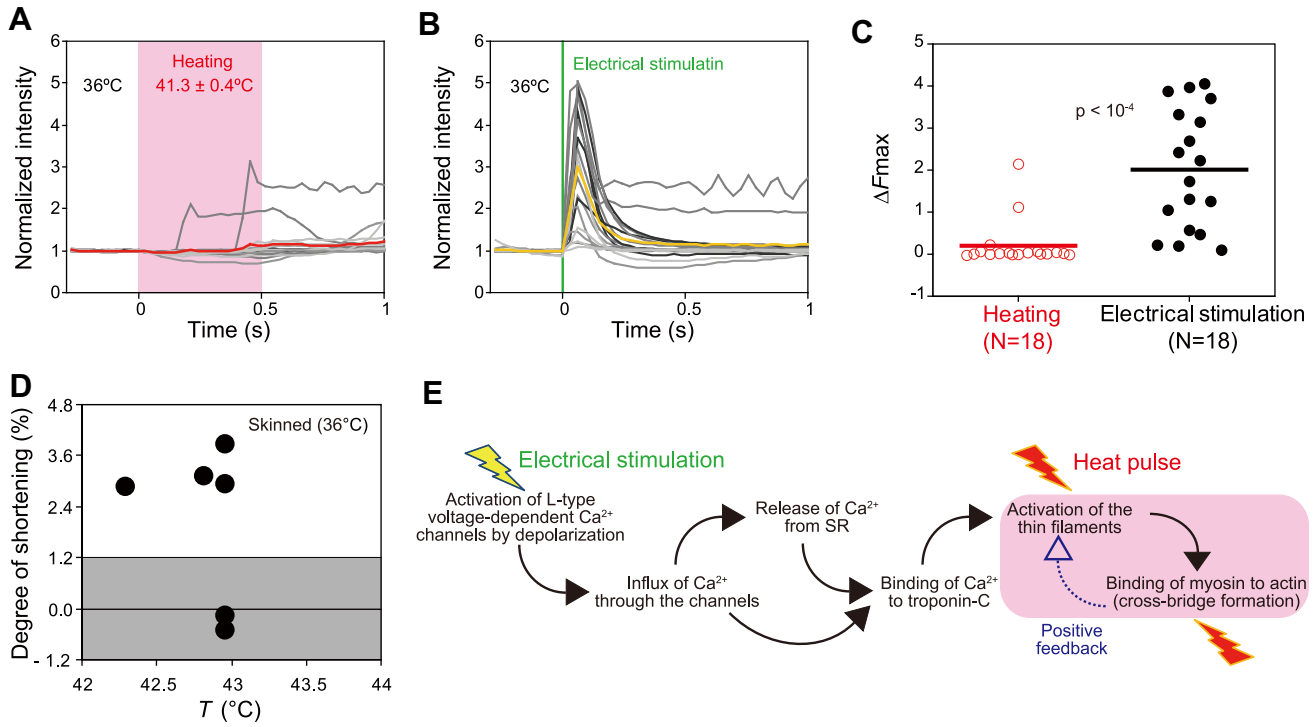

Fig. 4. Heat pulse-induced contraction occurs without Ca^{2+} transients. (A and B) Time courses of normalized fluorescence intensity of fluo-4 (Ca^{2+} indicator) in cardiomyocytes ($N = 18$ for either experiment). The cardiomyocytes were stimulated at time 0 by heating for 0.5 s (A, pink bar) or electrically (B, green bar). Red (A) and yellow (B) curves indicate the means of the time course of fluorescence intensity. (C) Proportion of the maximum changes in normalized fluorescence intensity, ΔF_{max}, induced by heat pulses (red) or electrical stimulation (black). Horizontal bars indicate the means. (D) A relationship between temperature during heating and the degree of shortening of skinned cardiomyocytes in the Ca^{2+}-free solution. Ambient temperature in A–D was 36 ± 0.5 °C. (E) A flow chart showing the pathway of excitation–contraction coupling in cardiomyocytes. Heat pulse induced contraction directly, by bypassing the processes related to Ca^{2+} signaling.

thermosensitive process (Fig. 4E). The affinity of tropomyosin on F-actin recovers when temperature returns [19], which should cause the relaxation of muscle.

Microscopic heat pulses revealed the thermosensitive contraction of cardiomyocytes without Ca^{2+} dynamics in excitation–contraction coupling. This non-invasive method has the potential to stimulate heart and skeletal muscles *in vivo* without Ca^{2+} transient, which differs from the conventional electrical stimulation. Decreased Ca^{2+} release from SR and reduced Ca^{2+} sensitivity of myofibrils cause muscle weakness [25]. The heat-pulse stimulation could be a useful means of activation of these muscles as the thermally induced contraction is independent of Ca^{2+} signaling. The thermosensitive contractions of cardiac muscles may also contribute to the understanding of the mechanism of abnormal contractions. A body temperature increase above 38 °C in young children causes uncontrolled muscle contractions, known as febrile seizure [26]. The thermosensitivity of muscle may operate as a part of such abnormal contractions. Identifications of muscle proteins with abnormal thermosensitivities, such as mutant tropomyosins with low dissociation temperature from F-actin, could further clarify the molecular mechanism of febrile seizure. Muscles are known to be a heat-generating system in addition to a contractile apparatus, but they are also a thermosensitive system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.015.

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