COMMUNICATION

Microscopic heat pulses activate cardiac thin filaments

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During the excitation–contraction coupling of the heart, sarcomeres are activated via thin filament structural changes (i.e., from the "off" state to the "on" state) in response to a release of Ca^{2+} from the sarcoplasmic reticulum. This process involves chemical reactions that are highly dependent on ambient temperature; for example, catalytic activity of the actomyosin ATPase rises with increasing temperature. Here, we investigate the effects of rapid heating by focused infrared (IR) laser irradiation on the sliding of thin filaments reconstituted with human α-tropomyosin and bovine ventricular troponin in an in vitro motility assay. We perform high-precision analyses measuring temperature by the fluorescence intensity of rhodamine-phalloidin–labeled F-actin coupled with a fluorescent thermosensor sheet containing the temperature-sensitive dye Europium (III) thenoyltrifluoroacetonate trihydrate. This approach enables a shift in temperature from 25°C to ∼46°C within 0.2 s. We find that in the absence of Ca^{2+} and presence of ATP, IR laser irradiation elicits sliding movements of reconstituted thin filaments with a sliding velocity that increases as a function of temperature. The heating-induced acceleration of thin filament sliding likewise occurs in the presence of Ca^{2+} and ATP; however, the temperature dependence is more than twofold less pronounced. These findings could indicate that in the mammalian heart, the on–off equilibrium of the cardiac thin filament state is partially shifted toward the on state in diastole at physiological body temperature, enabling rapid and efficient myocardial dynamics in systole.

Introduction

Contraction of cardiac muscle is induced by electric stimuli and the ensuing depolarization of the sarcolemma. Ca^{2+} enters the myocyte via sarcolemmal L-type Ca^{2+} channels that are located in the T-tubules (Bers, 2001, 2002; Kobirumaki-Shimozawa et al., 2014; Shimozawa et al., 2017 and references therein). This Ca^{2+} induces the transient release of Ca^{2+} from the SR (Ca^{2+} transient), resulting in the binding of Ca^{2+} to troponin C (TnC) on the thin filament. Ca^{2+} binding to TnC causes tropomyosin (Tm) to move across the thin filament surface, which promotes myosin attachment to actin (Solaro and Rarick, 1998; Fukuda et al., 2009; Kobirumaki-Shimozawa et al., 2014 and references therein). Cardiac myofilaments are not fully activated under physiological conditions, because (a) the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained relatively low {pCa (= −log $[Ca^{2+}]$) 6}, even at the peak of systole (Bers, 2001, 2002; Kobirumaki-Shimozawa et al., 2014; Shimozawa et al., 2017 and references therein), and (b) the working range of sarcomere length is on the shorter end of the resting distribution (hence lesser functionality of the Frank-Starling mechanism; Kobirumaki-Shimozawa et al., 2016).

During diastole, the C-terminal domain of TnI tightly binds to actin, and Tm blocks the actomyosin interaction ("off" state). However, when Ca^{2+} binds to the regulatory Ca^{2+}-binding site of TnC during systole, the C-terminal domain of TnI is dissociated from actin and binds to the N-terminal domain of TnC ("on" state). The on–off equilibrium of the thin filament state depends on the isoform of Tn subunits, as well as the number of strongly bound cross-bridges (Solaro and Rarick, 1998; Fukuda et al., 2009; Kobirumaki-Shimozawa et al., 2014 and references therein).

In the sarcomere, myosin molecules hydrolyze ATP in the presence of actin and convert chemical energy to generate myocardial dynamics. It is well established that this process involves chemical reactions that are highly dependent on ambient temperature; namely, the actomyosin ATPase rate is increased

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with increasing temperature (Bárány, 1967; de Tombe and Stienen, 2007). Accordingly, myocardial shortening and active force production (the former of which occurs during the ejection phase in the heart in vivo) are both enhanced with increasing temperature (Harrison and Bers, 1989; de Tombe and ter Keurs, 1990; Fujita and Kawai, 2002). However, as pointed out previously by Ranatunga (1994), in muscle mechanics studies, high active force produced at high temperatures is likely to cause irreversible damage to the sarcomere structure. Therefore, little information is available on the temperature dependence of the thick–thin filament sliding in myocardial preparations within the body temperature range.

Recently, we found that a rapid increase in temperature (within 0.2 s) via a water-absorbable 1,455-nm infrared (IR) laser beam causes reversible and reproducible shortening of intact isolated rat ventricular myocytes (Oyama et al., 2012). It is important to recognize that unlike the normal excitation–contraction coupling, this phenomenon is not preceded by Ca^{2+} transients; hence, it occurs in a Ca^{2+}-independent fashion. At a physiological baseline temperature of 36°C, the magnitude of myocyte shortening upon an increase in temperature (ΔT) of ~5°C was similar to that obtained under electric field stimulation (i.e., ~6%). This heating-induced contraction was blocked by the actomyosin inhibitor blebbistatin and occurs in skinned myocytes under the relaxing condition, thus indicating that cardiac cross-bridge cycling is promoted at temperatures of a few degrees (°C) higher than mammalian body temperature.

The present study was undertaken to investigate whether heating-induced actomyosin interaction occurs at the single-molecule level by taking advantage of the in vitro motility assay. We used filamentous actin (F-actin) and heavy meromyosin (HMM) extracted from rabbit fast skeletal muscle and recombinant human α-Tm (αTm) and bovine ventricular Tn. Experiments were performed in the range of 25°C to ~46°C, with or without Tm–Tn, in the absence and presence of Ca^{2+}. We showed that (a) IR laser irradiation-induced heating caused sliding behavior of reconstituted thin filaments (F-actin plus Tm–Tn) in the absence of Ca^{2+}, with an increase in velocity as a function of temperature, and (b) the temperature dependence was more than twofold less in the presence of Ca^{2+}, which is similar to that observed for F-actin. Although recent studies have revealed the complexity of the thin filament regulation involving cooperative interactions between actin filaments, myosin, Tm, Tn, and Ca^{2+} (e.g., Mijailovich et al., 2012), in order to clearly address the mechanistic implications, we discuss the present findings based on the two-state on–off model of thin filament regulation (e.g., Solaro and Rarick, 1998; Kobirumaki-Shimozawa et al., 2012). Accordingly, we suggest that during diastole in the mammalian heart in vivo, the on–off equilibrium of the cardiac thin filament is partially shifted toward the on state at body temperature. Hence, a small amount of Ca^{2+} released during the excitation–contraction coupling may effectively promote cross-bridge cycling.

Materials and methods

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 Regulations for Animal Experimentation at Waseda University. All experiments were performed at Waseda University.

Proteins

For this study, we used thin filaments composed of the bovine cardiac Tn complex, human Tm expressed in *Escherichia coli*, and rabbit fast skeletal actin. The recombinant wild-type Tm was expressed from human cardiac muscle αTm complementary DNA in *E. coli*. It was originally purified in the laboratory of Dr. Bryan Chase (Florida State University, Tallahassee, FL; e.g., Schoffstall et al., 2006; Wang et al., 2011). Because bacterially expressed proteins lack N-terminal acetylation, two extra amino acid residues, Gly and Ser, were attached to the N terminus to functionally substitute for the acetylation. Actin is one of the most conserved proteins known, and rabbit fast skeletal actin, which is easy to obtain in large quantities, differs from human cardiac actin by only five conservative residue changes (Table S1). We therefore used rabbit fast skeletal actin in the present study. Rabbits were purchased from Japan Laboratory Animals and used for the present study. Actin and HMM (α-chymotrypsin proteolytic fragment of myosin II) were purified from fresh fast skeletal muscles based on our previously described method (Fujita et al., 1996; Suzuki et al., 1996). Tn was extracted from fresh bovine ventricles, as described previously (e.g., Potter, 1982), which is, like actin, easy to obtain in a large quantity (prepared in the laboratory of Dr. Masataka Kawai, University of Iowa, Iowa City, IA). Bovine hearts were obtained at a local slaughterhouse. It is to be noted that (a) identities are all >90% between human and bovine Tn subunits (95%, 92%, and 99% for TnT, TnI, and TnC, respectively; Table S1), and (b) the presently used reconstituted thin filaments (F-actin plus human αTm-bovine Tn) exhibited a sliding velocity similar to that composed of rabbit fast skeletal F-actin, human αTm, and human Tn (Loong et al., 2013; measured with rabbit fast skeletal HMM in the presence of saturating [Ca^{2+}] at 30°C).

Solutions

The compositions of solutions used in the present study were as follows: (a) F-buffer: 2 mM $MgCl_2$, 1.5 mM NaN_3, 100 mM KCl, 10 mM dithiothreitol (DTT), and 2 mM MOPS, pH 7.0; (b) Relaxing (nonactivating) solution (−Ca^{2+}, pCa 9): 4 mM $MgCl_2$, 1 mM EGTA, 25 mM KCl, 10 mM DTT, and 25 mM imidazole-HCl; (c) activating solution (+Ca^{2+}, pCa 5): 1 mM EGTA, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 25 mM KCl, 10 mM DTT, and 25 mM imidazole- HCl. For all solutions except F-buffer, ionic strength was 50 mM, and the pH was adjusted to 7.40 with KOH at 25°C.

In vitro motility assay

Actin was polymerized in F-buffer at room temperature for 30 min, and 2.4 µM F-actin was labeled with rhodamine-phalloidin (7.2 µM; Molecular Probes) at 4°C overnight. The rhodamine-phalloidin F-actin was stored on ice (~3°C) and used within 2 wk. The reconstitution of thin filaments was performed in Eppendorf tubes (F-buffer, 20 µl) that contained 1.2 µM F-actin, 1.2 µM Tm, and 1.2 µM Tn on ice for ~1 h. Then, the annealing treatment (45°C for 10 min) was performed to achieve correct head-to-tail interaction between neighboring Tm molecules along the actin filament (Ishiwata, 1973) and improve the reproducibility of results. Afterwards, the tubes were stored on ice again.

The in vitro motility assay with HMM was performed based on our published procedures; namely, a coverslip (24 × 60 mm; Matsunami Glass) was sonicated in 0.45 M KOH for 15 min, acetone for 15 min, and ethanol for 15 min to remove dust and coating materials on the glass surface. The surface of the coverslip was then coated with 0.1% collodion dissolved in 3-methylbutyl acetate. The coverslips were dried at 25°C for ∼10 min, and further incubation at 50°C overnight improved data reproducibility. Another coverslip (18 × 18 mm; Matsunami Glass) was glued to the collodion-coated coverslip with double-sided tape to yield a flow-cell volume of ∼20 µl.

Because of the highly viscous nature of the HMM stock solution (100 mg/ml), it was diluted 10 times with relaxing solution, and the concentration was measured three times by absorptiometry (V-550ST; JASCO) before experimentation to avoid pipetting errors. The HMM solution (diluted to 30 µg/ml; 20 µl) was applied to one side of the flow cell and incubated for 60 s to attach HMM to the collodion-coated glass surface. Another drop of 20 µl HMM solution was applied to the flow cell from the other side and incubated for 60 s. Subsequently, 20 µl BSA solution (dissolved as 5 mg/ml in either relaxing or activating solution) was applied to the flow cell and allowed to settle for 5 min. Thereafter, a 50-µl drop of experimental solution containing 2 mM Na_2ATP, 1 mg/ml BSA, 5 nM F-actin, or reconstituted thin filaments was applied to the flow cell. When reconstituted thin filaments were used, excess amounts of Tm and Tn (both at 100 nM) were added to stabilize the structure of thin filaments by promoting the binding of these proteins to F-actin (Gordon et al., 1997). The solution also contained 25 mM glucose, 0.22 mg/ml glucose oxidase, and 0.036 mg/ml catalase to remove dissolved oxygen as well as to minimize photobleaching of rhodamine. After two open sides had been sealed with nonfluorescent enamel, the flow cell was placed under a microscope (see below). All of these preparations were performed at 25°C ± 1°C.

Optical setup

We used a fluorescence microscope described in detail in our previous studies (Oyama et al., 2012; Shintani et al., 2015). The optical setup was built around an inverted microscope IX70 (Olympus) with an objective lens (PlanApo N 60×/1.45 Oil; Olympus). Namely, a stable light source, Light Engine (with wavelengths of 549/15 nm and 377/50 nm; Lumencor), was used for excitation of actin filaments stained with rhodamine-phalloidin and a thermosensor sheet. A dichroic mirror (FF562-Di02; Semrock) and an emission filter (BA580IF; Olympus) were mounted. The solution was directly heated by focusing the IR-laser beam (λ = 1,455 nm, for 2 s; KPS-STD-BT-RFL-1455-02-CO; Keopsys) under the microscope. The laser power was 33 mW, which was measured at the top of the objective lens by using a thermal disk sensor and a power meter (LM-3 and FieldMaster; Coherent). The ON/OFF of heating was regulated by a shutter system (SSH-C4B; SIGMAKOKI) placed in the light path of the IR laser beam. Fluorescence images were recorded using an electron-multiplying charge-coupled device camera (iXon EM+ 897; Andor Technology) at 33 frames per second and stored in a Windows PC via ANDOR IQ software (Andor Technology). The present experimental setup is illustrated in Fig. 1 A.

Temperature measurement

The fluorescence intensity (FI) of 0.2 µM rhodamine-phalloidin in relaxing solution (with no ATP or BSA) was measured by a fluorescence spectrophotometer (F-4500; Hitachi High-Technologies) at various temperatures. The temperature in a cuvette was controlled by a precision thermostatic circulator (AB-1600; ATTO) and measured by a digital thermometer (ASF-250T; AS ONE). The excitation and emission wavelengths were 550 and 570–580 nm, respectively.

Temperatures induced by IR laser in flow cells were measured with rhodamine-phalloidin–labeled F-actin. The filaments were attached to HMM in relaxing solution without ATP for 30 min at room temperature. Temperature change was calculated based on the relative changes of FI (FI during heating divided by FI after heating). The backgrounds with or without IR laser irradiation were subtracted in this calculation.

Thermosensor sheets were prepared as follows: 5 mg/ml Europium (III) thenoyltrifluoroacetonate trihydrate (Eu-TTA; Acros Organics) and 10 mg/ml Poly(methyl methacrylate) (Sigma-Aldrich) in acetone (Wako) were spin-coated on a 35-mm glass-base dish (no. 3911–035; AGC Techno Glass) with a microcentrifugation (Capsulefuge PMC-060; Tomy Seiko). When normalized at 25°C, the temperature dependence of the FI was −2.8%/°C (Itoh et al., 2014). Solutions containing HMM and F-actin were applied to the thermosensor sheet. Temperature changes induced by the IR laser were measured at the same position of the thermosensor sheet and fluorescent F-actin. Room temperature (i.e., baseline temperature) was 25°C ± 1°C.

Data analysis

We manually tracked the filaments (either F-actin or reconstituted thin filaments) at various time intervals (on average 150–210 ms) and calculated the sliding velocity during each interval. Then, the data from all intervals were averaged to yield the sliding velocity.

We analyzed smooth movements of filaments (either F-actin or reconstituted thin filaments) in straight directions by using a plugin command (manual tracking) in ImageJ (National Institutes of Health). We defined the position of each filament at the onset of an interval and did not exclude the data of F-actin or reconstituted thin filaments crossing the 20-µm divisions. Two flow cells were used for each experimental condition, and the velocity measurements were performed on >50 filaments for each flow cell. We divided the distance from the laser center by 20 µm (up to 120 µm) and compared the sliding velocities of F-actin or reconstituted thin filaments obtained in various positions with different temperatures. Significant differences were assigned using the unpaired, two-sided *t* test as appropriate. Data are expressed as mean ± SEM, with *n* representing the number of intervals (see above). Statistical significance was assumed to be $P < 0.05$. NS indicates $P > 0.05$.

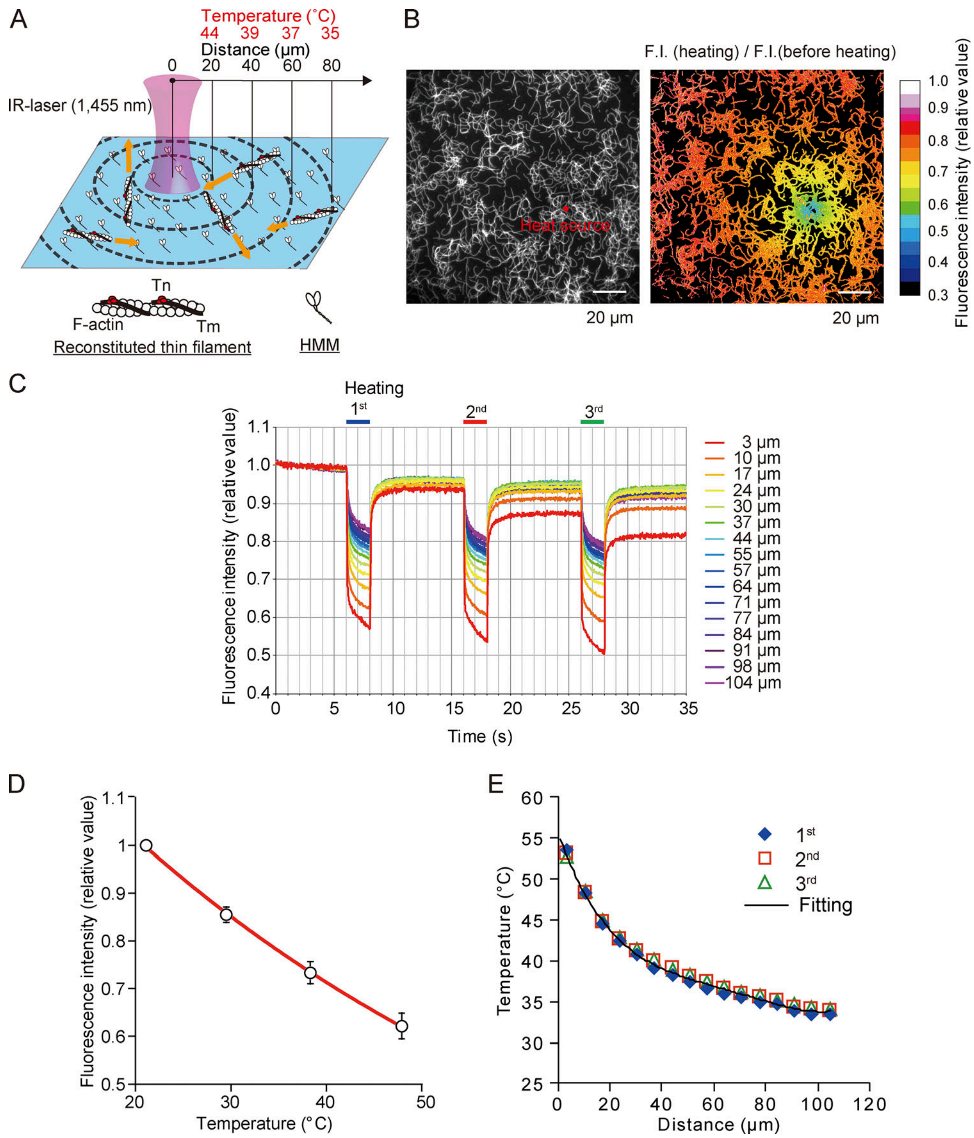

Figure 1. **IR laser–based local heating and temperature imaging system. (A)** Schematic illustration of the present experimental system. Reconstituted thin filaments (or F-actin) interacted with HMM attached to the glass surface. Reconstituted thin filaments and HMM are illustrated below. Temperature was directly increased by an IR laser beam (λ = 1,455 nm), which elicits thin filament movements in various directions (as shown by orange arrows), as a function of the distance from the heat source (distance shown by dashed gray circles from 0 to 80 µm, with temperatures denoted; cf. D). **(B)** Left: Fluorescence image of rhodamine-phalloidin–labeled F-actin filaments during heating. The IR laser was focused on a red point as indicated by "Heat source." Right: Ratio image showing a change in FI of F-actin before and during heating. Color map on the right indicates the magnitude of change. F-actin at a concentration of ∼10 nM was applied to the coverslip. **(C)** Time course of changes in FI of F-actin filaments located at various distances from the heat source (distances indicated on right). Heating with a duration of 2 s was applied three times in a consecutive manner at an interval of 10 s. Blue, first heating; red, second heating; and green, third heating. **(D)** Relationship between temperature and FI of rhodamine-phalloidin in the absence of F-actin. FI was measured by a fluorescence spectrophotometer. Red line is an exponential fitting curve; i.e., $Y = 1.45\ e^{-0.0177X}$. Data from measurements 1–4 in Fig. S1 were used. Number of measurements, 3. Data represent mean ± SEM. **(E)** Relationship of the distance from the heat source (denoted as "Distance" on the abscissa) vs. temperature. Data in C were analyzed based on a calibration curve in D. Temperature decreased as a function of the distance from the heat source, and therefore, data were fitted by the following polynomial function: $y = 6.39 \times 10^{-7} x^4 - 1.74 \times 10^{-4} x^3 + 1.76 \times 10^{-2} x^2 - 8.82 \times 10^{-1} x + 5.56 \times 10$. Note the high reproducibility of this relationship. Blue, first heating; red, second heating; and green, third heating (see C).

Online supplemental material

Fig. S1 shows the temperature dependence of the fluorescence intensity of rhodamine-phalloidin measured by a spectrophotometer. Fig. S2 compares temperatures at various distances from the heat source measured by rhodamine-phalloidin–labeled F-actin and a fluorescent thermosensor sheet. Fig. S3 shows the temperature dependence of sliding velocity of F-actin and reconstituted thin filaments with cardiac myosin at pCa 9. Fig. S4 shows the temperature dependence of sliding velocity of F-actin and reconstituted thin filaments with cardiac myosin at pCa 5. Table S1 compares the amino acid sequences of proteins used in the present study and those of corresponding human cardiac proteins.

Results and discussion

First, we quantified changes in local temperatures by IR laser (λ = 1,455 nm; water-absorbable wavelength) irradiation in flow cells using rhodamine-phalloidin–labeled F-actin filaments (Kato et al., 1999; Fig. 1 B). F-actin filaments were interacted with HMM in rigor solution (i.e., relaxing solution with no ATP) in the flow cell for 30 min at room temperature; hence, no sliding movement occurred. After laser irradiation, FI for F-actin proximal to the laser center (less than ∼20 µm) did not return to the baseline level and decreased sequentially with repeated irradiation (Fig. 1 C). This FI reduction is likely caused by dissociation of rhodamine-phalloidin from F-actin (see De La Cruz and Pollard, 1996), because we confirmed, by using a fluorescence spectrophotometer, that FI for rhodamine-phalloidin returned to the preirradiation level when the temperature increased from 21.1°C to 47.9°C and then decreased to 21.6°C (Fig. S1). Therefore, an increase in temperature up to ∼48°C does not irreversibly disrupt the fluorescence characteristics of rhodamine-phalloidin under the present experimental condition. For high-precision analyses of a change in temperature, we calculated it based on the temperature sensitivity measured in solution by a fluorescence spectrophotometer (Figs. 1 D and S1). The irreversible FI reductions were compensated via differences in FI before and after heating (Fig. 1 E). We confirmed that the temperatures measured by fluorescent F-actin were similar to those measured by fluorescent thermosensor sheets (Fig. S2).

We applied IR laser irradiation to F-actin at pCa 9 (+ATP). The sliding velocity of F-actin on the HMM-coated glass surface was rapidly (less than ∼30 µm/s) increased during heating (Fig. 2 A, top; Video 1), consistent with our earlier findings using F-actin on myosin-coated glass (Kato et al., 1999) and microtubules on kinesin-coated glass (Kawaguchi and Ishiwata, 2001). A similar finding was obtained in the in vitro motility assay on the porcine ventricular myosin-coated glass (Fig. S3 and Video 3; see online supplemental material for details). The heating effect was more pronounced near the heat source (Fig. 2 B, top), indicating that the sliding velocity increased as a function of temperature. Provided that the actomyosin ATPase rate is increased with increasing temperature in cardiac muscle (see Bers, 2001 and references therein), the increase in the sliding velocity is likely coupled to the acceleration of the cross-bridge cycling rate (compare Bárány, 1967; Anson, 1992). Here, the sliding velocity was decreased to the baseline level (i.e., ∼5 µm/s) immediately (less than ∼1 s) upon cessation of the laser irradiation (Fig. 2 B, top). The sliding velocity of F-actin, with and without heating, was consistent with that observed earlier by us under a similar experimental condition in which IR laser pulses were applied to raise the temperature for a shorter period of time (0.5 s) at a faster rate (within 10 ms; Kato et al., 1999), indicating that a rapid increase in temperature up to ∼40°C using an IR laser does not cause thermal denaturation of proteins in the in vitro motility assay (as long as the heating duration is sufficiently short, as in the current study). In the present study, F-actin filaments proximal to the heat source (less than ∼10 µm) stopped moving after the first laser irradiation and did not resume moving upon subsequent irradiation (Video 1). Given the close proximity to the heart source, we consider that this irreversibility is coupled with the thermal denaturation of HMM (cf. Shriver and Kamath, 1990).

Next, we investigated whether or not and how reconstituted thin filaments (F-actin plus αTm–Tn) respond to rapid heating at pCa 9 (+ATP). While reconstituted thin filaments did not move at 25°C, laser irradiation elicited thin filament sliding, with a velocity of ∼30 µm/s near the heart source (Fig. 2, A and B, bottom; Video 2; as in the case of F-actin; cf. Fig. 2, A and B, top). A similar finding was obtained in the in vitro motility assay on porcine ventricular myosin-coated glass (Fig. S3 and Video 4; see online supplemental material for details). The sliding velocity tended to be faster for F-actin than reconstituted thin filaments within the distance range of ∼20–100 µm from the heat source (Fig. 3, A and B); however, the velocity became similar at less than ∼20 µm. The plot of sliding velocity vs. temperature revealed that F-actin moved significantly faster than reconstituted thin filaments within the temperature range of ∼35–41°C, and the difference became smaller with increasing temperature (Fig. 3 C). At the highest temperature we detected (∼46°C), the sliding velocity became similar for F-actin and reconstituted thin filaments (i.e., ∼21 µm/s). These findings of reconstituted thin filaments were in good agreement with our previous results on intact rat ventricular myocytes (Oyama et al., 2012) in that IR laser pulses caused reversible Ca^{2+}-independent shortening in the temperature range of ∼40–43°C. The temperature coefficient (Q_{10}) values were 2.4 and 5.5 for F-actin and reconstituted thin filaments, respectively (Fig. 3 D).

It should be stressed that in the mammalian body temperature range (∼36–38°C), moderate sliding movements were observed with velocities of 8.3 ± 0.7 and 8.6 ± 0.5 µm/s at ∼36 and ∼38°C, respectively (∼40% compared with the maximal velocity at ∼46°C for reconstituted thin filaments or F-actin with rabbit fast skeletal HMM; Fig. 3 C). When bovine ventricular myosin was used, the thin filament sliding velocities were ∼20% at ∼37 and ∼38°C compared with the maximal velocity at ∼40°C for F-actin (Fig. S3). These findings with rabbit fast skeletal HMM and bovine ventricular myosin are consistent with our earlier result from rat ventricular myocytes (Oyama et al., 2012) that exhibited moderate shortening of ∼2.5% at the body temperature of rats (∼38°C; maximal shortening, ∼6%). A similar phenomenon was reported by Ranatunga (1994) in intact rat extensor digitorum longus and skinned rabbit psoas muscles; viz., heating of solution to body temperature caused partial activation at rest in a reversible manner. This effect was sarcomere length dependent (i.e., more pronounced at a longer length) and in skinned rabbit psoas muscle reached ∼80% of Ca^{2+}-activated force at a sarcomere length of 3.0 µm at 40°C. Given a marked depressant effect of inorganic phosphate (10 mM) on the heating-induced rise in force, an actomyosin interaction is likely to underlie this phenomenon. We therefore consider that mammalian thin filaments, either cardiac or skeletal, are partially activated under the relaxing condition at body temperature.

We then investigated the effects of laser irradiation on F-actin or reconstituted thin filaments at pCa 5 (+ATP). As observed at pCa 9, laser irradiation increased the sliding velocity for both F-actin and reconstituted thin filaments, of which the

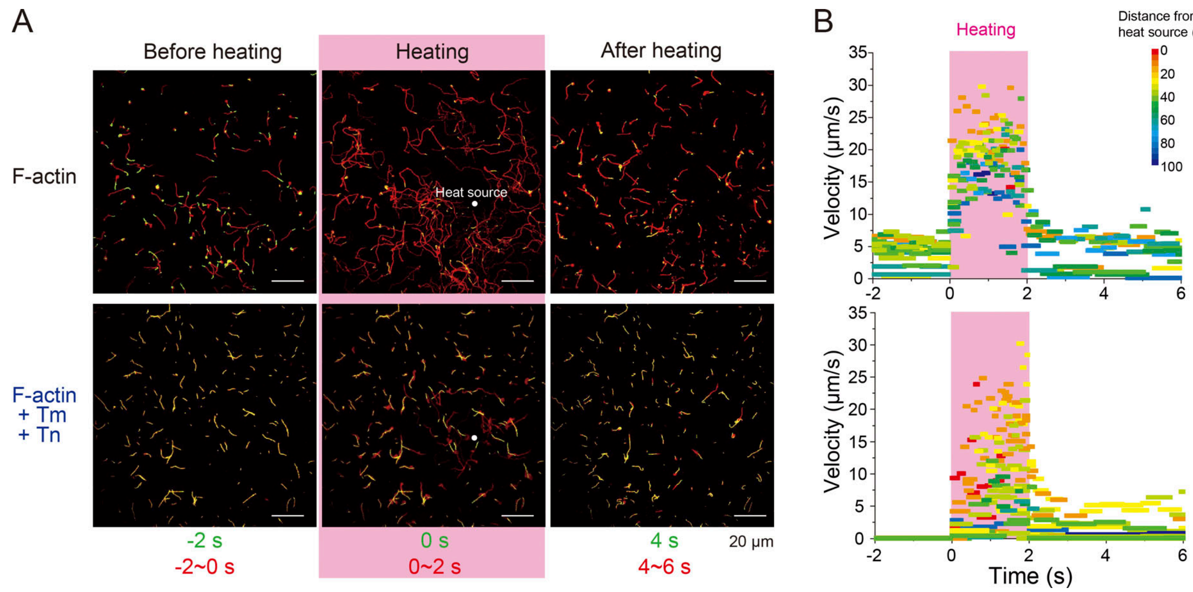

Figure 2. **Thermal activation of F-actin and reconstituted thin filaments at pCa 9. (A)** Effects of IR laser irradiation on the motility of F-actin (top) or reconstituted thin filaments (bottom). Merged fluorescence images before (left), during (middle), and after (right) heating are shown. Heat source is indicated as a small white circle (denoted as "Heat source") in the middle panels marked in pink. Images show 2 s before heating, at the onset of heating, and 2 s after heating, respectively, in the left, middle, and right panels (as indicated on the bottom in green). Images in red represent trajectories of the movement of filaments during 2 s (as indicated on bottom). Images in yellow represent immobile filaments. See Videos 1 and 2 for F-actin and reconstituted thin filaments, respectively. Scale bars, 20 µm. **(B)** Time courses of sliding velocity of F-actin (top) or reconstituted thin filaments (bottom) located at various distances from the heat source (see color map on right) before, during, and after heating. Heating was given for 2 s from 0 to 2 s (marked in pink). Note similar sliding velocities were obtained upon heating for F-actin and reconstituted thin filaments.

effects were more pronounced proximal to the heat source (Fig. 4, A and B). A similar finding was obtained in the in vitro motility assay on porcine ventricular myosin-coated glass (Fig. S4; see online supplemental material for details). At 25°C, the sliding velocity was slightly faster for reconstituted thin filaments (4.5 ± 0.05 and 6.4 ± 0.1 µm/s [$P < 0.001$] for F-actin and reconstituted thin filaments, respectively; Fig. 4 C). The difference in sliding velocity between F-actin and reconstituted thin filaments at 25°C was qualitatively similar to that observed previously by Homsher et al. (2003) using Tm and Tn from bovine ventricular muscle and myosin from rabbit fast skeletal muscle. Interestingly, in contrast to the data at pCa 9, no significant difference was observed between groups at higher temperatures. In comparison with the data on reconstituted thin filaments at pCa 9, the sliding velocity was faster at pCa 5 than at pCa 9 and at and below mammalian body temperature (i.e., from 25°C to ∼38°C), and it reached a plateau at ∼41°C. At ∼41°C and ∼45°C, no significant difference was observed in the sliding velocity of reconstituted thin filaments at pCa 5 and 9. While Q_{10} was 2.6 for F-actin (similar to the value at pCa 9 [2.4]), it was 1.9 for reconstituted thin filaments (Fig. 4 D), demonstrating a marked decrease in the temperature sensitivity of thin filaments as compared with that at pCa 9 (i.e., Q_{10} 5.5; cf. Fig. 3 D).

What is the molecular mechanism by which rapid heating induces reversible sliding movement of thin filaments at pCa 9? We consider that the on–off equilibrium of the thin filament is susceptible to a change in ambient temperature, especially within the body temperature range; viz., an increase in temperature will shift the equilibrium toward the on state, and vice versa. Indeed, Tanaka and Oosawa (1971) demonstrated that Tm dissociates from F-actin with increasing temperature (i.e., at greater than ∼40°C). Later, Ishiwata (1978) demonstrated that Tn increases the affinity of Tm for F-actin, thereby stabilizing the thin filament structure, of which the effect is dependent on the Ca^{2+} concentration; viz., the thin filament structure is stabilized (destabilized) in the absence (presence) of Ca^{2+}. Further, Ishiwata (1978) investigated the effects of Tn subunits (i.e., TnT, TnI, and TnC) on the temperature-dependent dissociation of Tm from F-actin and found that TnT, but not TnI or TnC, markedly increases the dissociation temperature (i.e., from 36.5 to 46.5°C), with or without Ca^{2+}. Likewise, experiments on reconstituted thin filaments (F-actin plus Tm and the whole Tn) showed that Tm–Tn dissociates from F-actin as a function of temperature; namely, Tm–Tn starts to partially dissociate from F-actin at ∼38°C, with dissociation temperatures of 48.8°C and 47.0°C in the absence and presence of Ca^{2+}, respectively. Although in these previous studies different preparations (all proteins prepared from rabbit fast skeletal muscle) were used in solution in the absence of myosin, the heating-induced partial dissociation of Tm–Tn from F-actin (or weakening of the binding of Tm–Tn to F-actin) may account for activation of thin filaments at pCa 9, either in the present in vitro motility assay (Figs. 2, 3, and S3) or in cardiomyocytes (Oyama et al., 2012). This is because rapid heating by less than ∼10°C beyond mammalian body temperature (maximum ∼46°C for rabbit fast skeletal HMM in the present study) is unlikely to cause denaturation of proteins, especially myosin (whose subfragment 1 [S1] reportedly starts to show denaturation at 48°C at equilibrium; see Shriver and

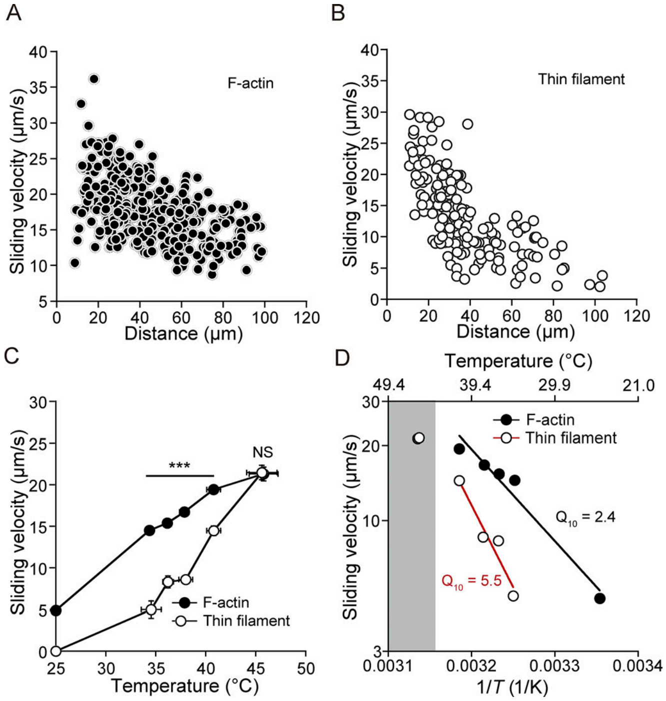

Figure 3. **Temperature dependence of sliding velocity of F-actin and reconstituted thin filaments at pCa 9. (A)** Relationship between the distance from the heat source and sliding velocity for F-actin. **(B)** Same as in A for reconstituted thin filaments. **(C)** Sliding velocity plotted against temperature. Closed circles, F-actin; open circles, reconstituted thin filaments. Sliding velocities were compared at 25°C (baseline temperature) and at higher temperatures raised by IR laser irradiation. Data represent mean ± SEM for both the x and y axes. Sliding velocities at 25°C are 4.9 ± 0.1 µm/s (n = 513) and ∼0 µm/s for F-actin and reconstituted thin filaments, respectively. Those at higher temperatures are as follows: 14.5 ± 0.4 (n = 32) and 5.0 ± 1.0 µm/s (n = 6) at 34.4 ± 0.4 and 34.5 ± 1.0°C, 15.4 ± 0.4 (n = 65) and 8.3 ± 0.7 µm/s (n = 20) at 36.1 ± 0.4 and 36.2 ± 0.5°C, 16.7 ± 0.4 (n = 92) and 8.6 ± 0.5 µm/s (n = 26) at 37.9 ± 0.5 and 38.0 ± 0.7°C, 19.4 ± 0.4 (n = 120) and 14.5 ± 0.6 µm/s (n = 89) at 40.8 ± 0.7 and 40.8 ± 0.7°C, and 21.3 ± 0.8 µm/s (n = 39) and 21.5 ± 0.9 µm/s (n = 28) at 45.8 ± 1.4 and 45.6 ± 1.5°C for F-actin and reconstituted thin filaments, respectively. ***, $P < 0.001$ for the y axis between groups (no significant differences present on the x axis for each comparison). **(D)** Arrhenius plot of sliding velocity for F-actin and reconstituted thin filaments. T, absolute temperature. The average values from C were used. Data obtained in the highest temperature range (shown in gray) were not employed for the analysis due to possible denaturation of myosin (see Shriver and Kamath, 1990). Sliding velocity (V) and temperature were expressed in logarithm. F-actin: $V = \exp(30.06 - 8{,}468/T)$ (R = 0.96). Reconstituted thin filaments: $V = \exp(50.96 - 15{,}161/T)$ (R = 0.98). Closed circles with a black solid line, F-actin; open circles with a red solid line, reconstituted thin filaments. Q_{10}, 2.4 (25–41°C) and 5.5 (34–41°C) for F-actin and reconstituted thin filaments, respectively.

Kamath, 1990); rather, it is likely to cause partial and reversible opening of the myosin-binding site of actin in thin filaments, thereby shifting the on–off equilibrium of the thin filament state toward the on state.

We observed continuous sliding movements of reconstituted thin filament at pCa 9 after cessation of IR laser irradiation near the heat source (∼20 µm; Fig. 2, A and B; and Video 2). Provided that (a) the dissociation temperature is reportedly 46.5°C for Tm–Tn from F-actin (see above; although Tm and Tn were different from those used by Ishiwata, 1978), and (b) the temperature increased from ∼45°C to ∼55°C as the distance from the heat source decreased from ∼20 to ∼0 µm (Fig. 1 E), we consider that Tm–Tn is fully dissociated from F-actin, resulting in continuous sliding movements. However, within the physiological body temperature range (i.e., 40–80 µm; see Fig. 1, A and B; and Video 2), no such movements were observed, indicating reversibility of the effect of IR laser irradiation (under these settings). Therefore, the observed on–off regulation of thin filament sliding is unlikely elicited by full dissociation of Tm–Tn from F-actin and its subsequent reassociation to F-actin, beyond ∼20 µm from the heat source (compare Ranatunga, 1994).

Earlier, de Tombe and ter Keurs (1990) investigated the effect of temperature on the maximal unloaded shortening velocity (V_{max}, which is similar to thin filament sliding in the in vitro motility assay, as in Toyoshima et al., 1987; Brizendine et al., 2015) in intact rat ventricular trabeculae during twitch in the presence of 1.5 mM extracellular Ca^{2+} concentration. They varied the temperature within the range of 20–30°C, and measured V_{max} when sarcomere length was shortened from 2.2 to 1.9 µm with active force clamped during shortening. Accordingly, they obtained a Q_{10} of 4.6–4.9, which is similar to that observed for reconstituted thin filaments in the present study at pCa 9 (i.e., 5.5 and 4.4 for rabbit fast skeletal HMM [Fig. 3 D] and porcine ventricular myosin [Fig. S3 D], respectively). Later, de Tombe and Stienen (2007) yielded a Q_{10} of 3.3 for the rate of active force redevelopment (k_{tr}; equal to cross-bridge attachment rate [f] + detachment rate [g]; see Huxley, 1957; Brenner and Eisenberg, 1986; Metzger et al., 1989; Terui et al., 2008) of maximal force in skinned rat ventricular trabeculae at 15–25°C (similar to the result in Hancock et al., 1996). These authors provided evidence that, contrary to g, which is insensitive to temperature, f varies as a function of temperature, following Ca^{2+} binding to TnC. In contrast, a previous study, which took advantage of in vitro motility and actin-activated ATPase assays with rabbit fast skeletal muscle and nonmuscle HMM, demonstrated that the ADP release step (i.e., g) varies as a function of temperature (Yengo et al., 2012). Therefore, future studies are needed to systematically investigate if f, g, or both are sensitive to a change in temperature in vitro and in muscle preparations from the heart of various animal species (see, e.g., Offer and Ranatunga, 2015; Rahman et al., 2018 on skeletal muscle).

It is worthwhile noting that compared with the previous findings on myocardial preparations, Q_{10} for reconstituted thin filaments obtained in the present study at pCa 5 was relatively small (i.e., 1.9), similar to the value for F-actin (Fig. 4 C), when rabbit fast skeletal HMM was used. This is presumably because the present experiments were performed at higher temperatures (i.e., up to ∼46°C), taking advantage of the in vitro motility assay. It has been reported in intact rat fast and slow skeletal muscles that Q_{10} for V_{max} varies markedly depending on the temperature range; viz., 2.4 and 1.8 for fast, and 3.5 and 2.0 for slow muscles, respectively, for the ranges 10–20°C and 25–35°C (Ranatunga, 2018). Given the temperature-sensitive nature of the Tm–Tn complex (Tanaka and Oosawa, 1971; Ishiwata, 1978), we interpret the difference in Q_{10} at pCa 5 and 9 to be explained as follows (i.e., 1.9 and 5.5 at pCa 5 and 9 for rabbit fast skeletal HMM, respectively [Figs. 3 and 4], and 3.5

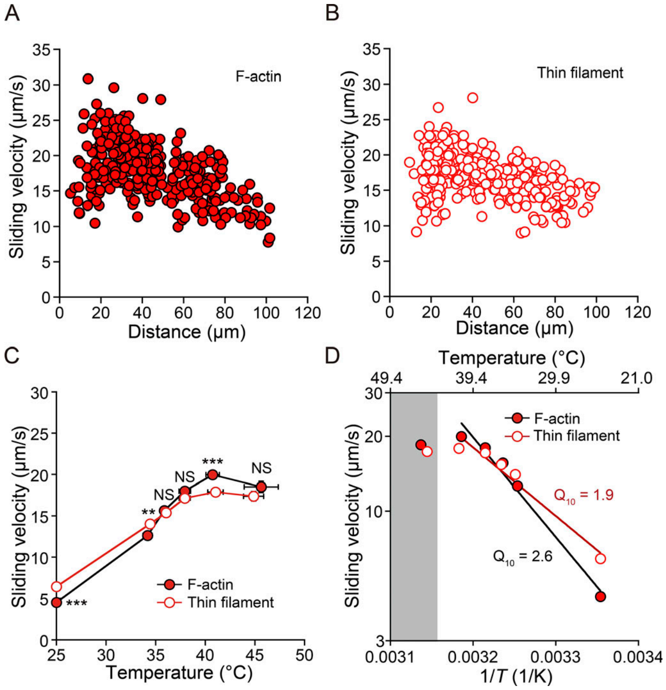

Figure 4. **Temperature dependence of sliding velocity of F-actin and reconstituted thin filaments at pCa 5. (A)** Relationship between the distance from the heat source and sliding velocity for F-actin. **(B)** Same as in A for reconstituted thin filaments. **(C)** Sliding velocity plotted against temperature. Closed circles, F-actin; open circles, reconstituted thin filaments. Sliding velocities were compared at 25°C (baseline temperature) and at higher temperatures raised by IR laser irradiation. Data represent mean ± SEM for both the x and y axes. Sliding velocities at 25°C are 4.5 ± 0.05 µm/s (n = 461) and 6.4 ± 0.1 µm/s (n = 352) for F-actin and reconstituted thin filaments, respectively. Those at higher temperatures are as follows: 12.6 ± 0.4 (n = 21) and 14.0 ± 0.3 µm/s (n = 32) at 34.2 ± 0.5 and 34.4 ± 0.4°C, 15.6 ± 0.3 (n = 69) and 15.4 ± 0.3 µm/s (n = 61) at 35.9 ± 0.3 and 36.1 ± 0.4°C, 18.0 ± 0.4 (n = 69) and 17.1 ± 0.3 µm/s (n = 72) at 37.9 ± 0.6 and 37.9 ± 0.6°C, 20.0 ± 0.3 (n = 119) and 17.9 ± 0.3 µm/s (n = 112) at 40.7 ± 0.7 and 41.0 ± 0.7°C, and 18.5 ± 0.7 (n = 45) and 17.4 ± 0.6 µm/s (n = 38) at 45.6 ± 1.7 and 44.9 ± 1.0°C for F-actin and reconstituted thin filaments, respectively. **, P < 0.01; ***, P < 0.001 for the y axis between groups (no significant differences were present on the x axis for each comparison). **(D)** Arrhenius plot of sliding velocity for F-actin and reconstituted thin filaments. T, absolute temperature. The average values from C were used. Data obtained in the highest temperature range (shown in gray) were not employed for the analysis (see Fig. 3 legend). Sliding velocity (V) and temperature were expressed in logarithm. F-actin: $V = \exp(32.65 - 9{,}269/T)$ (R = 0.97). Reconstituted thin filaments: $V = \exp(23.24 - 6{,}358/T)$ (R = 0.96). Closed circles with a black solid line, F-actin; open circles with a red solid line, reconstituted thin filaments. Q_{10} (25–41°C), 2.6 and 1.9 for F-actin and reconstituted thin filaments, respectively.

and 4.4 at pCa 5 and 9 for bovine ventricular myosin, respectively [Figs. S3 and S4]); at pCa 5, the on–off equilibrium of the thin filament state is shifted largely toward the on state, and therefore, the effect of heating-induced partial dissociation of Tm–Tn from F-actin is limited, resulting in a relatively small value of Q_{10}. While at pCa 9, the equilibrium is almost at the off state due to the fully suppressed actomyosin interaction by Tm–Tn, and therefore, the partial dissociation of Tm–Tn promotes a large fraction of myosin attaching to thin filaments and subsequent cross-bridge cycling, as evident in the present in vitro motility assay (Figs. 2, 3, S3, and S4) or intact cardiomyocytes (Oyama et al., 2012). Therefore, at physiological body temperature of mammals (e.g., ∼37°C in humans), the on–off equilibrium of the cardiac thin filament state is partially shifted toward the on state even in diastole; therefore, dynamic myocardial movements are effectively elicited in response to Ca^{2+} release from the SR, despite the relatively low concentration (pCa ∼6.0 even at the peak of systole; e.g., Bers, 2001, 2002; Kobirumaki-Shimozawa et al., 2014; Shimozawa et al., 2017 and references therein). Indeed, it has been reported in skinned cardiac muscles that Ca^{2+}-dependent sarcomere dynamics (cross-bridge cycling rate [e.g., Fitzsimons et al., 2001; Stelzer et al., 2006], rate of rise of isometric force [e.g., Terui et al., 2010], and apparent Ca^{2+} sensitivity [e.g., Fitzsimons and Moss, 1998; Fukuda et al., 1998, 2000; Terui et al., 2010]) is enhanced via cooperative activation when thin filaments are partially activated by strongly bound cross-bridges, such as NEM-S1 or the intrinsic actomyosin–ADP complex. We therefore propose that the role of partially activated thin filaments, by either heating or strongly bound cross-bridges or both, is an intrinsic nature of cardiac muscle to maximize the efficiency of Ca^{2+}-dependent sarcomeric activation at the low concentration of pCa ∼6.0. Future studies are warranted by investigating the effects of temperature within the body temperature range on mechanical properties of myocardial preparations by varying the Ca^{2+} concentration.

Earlier, Brunet et al. (2012) conducted in vitro motility assays with F-actin, human αTm, cardiac Tn, and rabbit fast skeletal HMM, with the temperature increased up to an unphysiological ∼63°C. In their study, the temperature was increased by microfabricated thermoelectric controllers (in ∼50 s to reach the steady-state level) from the baseline temperature of ∼20°C to 30°C. It was reported that F-actin and reconstituted thin filaments under the activation condition (pCa 5) exhibited acceleration of sliding movement upon an increase in temperature up to ∼60°C. At pCa 9, reconstituted thin filaments started to move at ∼45°C, and the sliding velocity remained increased with increasing the temperature up to ∼60°C. At ∼60°C, the sliding velocity became similar for F-actin and reconstituted thin filaments at pCa 5 and 9. The well-known study of Shriver and Kamath (1990) demonstrated that S1 and S2 of rabbit fast skeletal myosin (which was used in Brunet et al., 2012 and in the present study) became denatured at temperatures above 48°C and 41°C at equilibrium (i.e., after a long period of time), respectively. Therefore, due to denaturation of myosin ATPase, when the temperature is slowly increased (in ∼50 s in Brunet et al., 2012), actomyosin interaction unlikely occurs at ∼50°C and higher. We consider that the experimental solution temperature should have been lower than that estimated in Brunet et al. (2012). In their system, the temperature was increased by thin nickel film heaters and calculated via a gold-resistive thermometer that had been microfabricated on the inner face of the flow cell (distance from the inner face to the solution, ∼500 µm). Therefore, a large difference of thermal conductivity for gold vs. water (∼300 and ∼0.6 W/m/K for gold and water, respectively; National Astronomical Observatory of Japan, 2016) is likely to result in overestimation of the solution temperature.

Conclusion

In conclusion, we found that rapid heating induced thin filament sliding at pCa 9 in the in vitro motility assay using rabbit fast skeletal HMM or porcine ventricular myosin, and the sliding velocity was increased as a function of temperature. The heating-induced acceleration of thin filament sliding was likewise observed at pCa 5; however, the temperature dependence was less pronounced, regardless of the type of myosin. Given the present findings and those of our previous study on intact cardiomyocytes (Oyama et al., 2012), we conclude that mammalian cardiac thin filaments are partially activated in diastole at physiological body temperature, which facilitates rapid and efficient myocardial dynamics in response to Ca^{2+} release from the SR during the excitation–contraction coupling in systole.

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The authors declare no competing financial interests.

Author contributions: S. Ishii, K. Oyama, H. Itoh, S.A. Shintani, M. Suzuki, N. Fukuda, and S. Ishiwata conceived and designed the experiments. S. Ishii and K. Oyama performed experiments. S. Ishii, K. Oyama, and T. Arai analyzed the data. S. Ishii, K. Oyama, F. Kobirumaki-Shimozawa, T. Terui, N. Fukuda, and S. Ishiwata interpreted the data, drafted the manuscript, and revised it critically for intellectual concepts and content. All authors have approved the final draft of the manuscript, and their contributions qualify them as authors.

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