Mechanisms underlying hypothermia-induced cardiac contractile dysfunction


Rewarming patients from accidental hypothermia is often complicated by cardiovascular instability ranging from minor cardiac output depression to fatal circulatory collapse, the latter termed “rewarming shock.” However, the pathophysiological mechanisms of posthypothermic cardiovascular instability remain elusive, leaving mortality after accidental hypothermia between 50% and 80% (21, 23).

Experimental hypothermia research has disclosed that hypothermia may induce cardiac dysfunction (4), and, depending on level of severity, cardiac contractile dysfunction will be an essential determinant in cardiovascular instability during rewarming. Thus, the determination of the pathophysiological mechanisms underlying hypothermia-induced cardiac dysfunction will be critical to comprehensively treating hypothermia-induced myocardial failure in the clinical setting.

The present study focused on possible hypothermia-induced changes in regulatory processes at the level of cardiac sarcomeric proteins. It is well known that the functional dynamics of cardiac troponin C (cTnC) is not only dominated by the reception of Ca2+ but also the transduction of this Ca2+-binding signal by cardiac troponin I (cTnI) and cardiac troponin T (cTnT) (28, 29). Phosphorylation of these sarcomeric proteins tunes the intensity and dynamics of cardiac contraction and relaxation independent of membrane Ca2+ fluxes to meet physiological demands (28, 29). The phosphorylation of cTnI at Ser23/24 represents a well-established physiological mechanism for reduced myofilament Ca2+ sensitivity after β-adrenergic stimulation and the activation of PKA. Previous studies (7, 10, 11, 34, 41, 43–45) have reported that changes in the cTnI phosphorylation status of myofibrillar proteins underlie myocardial contractile dysfunction under various pathophysiological conditions, such as ischemia-reperfusion injury, hypertrophy, sepsis, and heart failure. A prevailing notion is that physiological states reflect a homeostatic distribution of phosphorylation of sarcomeric proteins, whereas pathophysiological states reflect a disturbance in this homeostasis with maladaptive distribution of these phosphorylations (27). Accordingly, we hypothesized that hypothermia and rewarming lead at least to changes in cardiac myofilament Ca2+ sensitivity and/or in the phosphorylation level of cTnI, thus contributing to reduced contractility.

To test this hypothesis, intact left ventricular (LV) rat papillary muscles were loaded with fura-2 AM to follow changes in the intracellular Ca2+ concentration ([Ca2+]i), and twitch force was evaluated in papillary muscle preparations maintained at 15°C for 1.5 h before being rewarmed to baseline temperature (30°C). Hypothermia at 15°C was chosen to mimic the core body temperature (15°C) of rats used in our previous in vivo rewarming shock experiments (16, 17, 37). In skinned papillary muscle fibers, we assessed maximal Ca2+-activated force (Fmax) and Ca2+ sensitivity of force in skinned papillary muscle fibers, Fmax was decreased by ~30%, whereas the pCa required for 50% of Fmax to reduced by 10.220.32.246 on August 15, 2017 http://ajpheart.physiology.org Downloaded from by 10.220.32.246 on August 15, 2017 http://ajpheart.physiology.org Downloaded from

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of Mayo Clinic approved the experimental protocols and care of the animals. Adult male Sprague-Dawley rats (250–350 g) were injected intramuscularly with ketamine (60 mg/kg) and xylazine (2.5 mg/kg) for anesthesia.

Isolation of LV papillary muscle. Hearts from anesthetized rats were quickly removed and placed in a custom-made dish continuously circulated with aerated Krebs-Henseleit (KH) solution containing (in mM) 118.4 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 10 glucose. Papillary muscles from the LV were gently excised and immediately mounted between a force transducer and a length change manipulator in a quartz cuvette continuously perfused with aerated KH solution (Guth Muscle Research System) (14). The average muscle length was 3 mm and diameter was ~1.2 mm, allowing for proper gas exchange with the perfusate (26). Force normalized to cross-sectional area (CSA) was expressed as milliNewtons per millimeter squared.

Address for reprint requests and other correspondence: G. C. Sieck, Dept. of Physiology and Biomedical Engineering, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905 (e-mail: sieck.gary@mayo.edu).
**Simultaneous measurement of intracellular Ca^{2+} transients and twitch force.** The horizontally mounted papillary muscle was loaded with 10 μM fura-2 AM for 1.5 h in oxygenated KH solution containing Pluronic F-127 [0.01% (wt/vol)], which facilitated the loading of fura-2 AM into the muscle fibers at room temperature (20–22°C) (26, 42). After being loaded, the muscle was washed out for 10 min and continuously perfused with KH solution aerated with 95% O_{2}-5% CO_{2} throughout the experiment. Afterward, the muscle was electrically stimulated at 0.5 Hz at the baseline temperature of 30°C, during which muscle length was adjusted for maximal twitch force. During hypothermia at 15°C, the papillary muscle was stimulated at 0.25 Hz to imitate the hypothermia-induced change in heart rate of the intact heart at this temperature. Twitch force and the corresponding ratio signals from fura-2 fluoroscences at 340- and 380-nm excitation were recorded using a real-time data-acquisition program (LabVIEW, National Instruments, Austin, TX). To minimize photobleaching, the excitation shutter was only opened during data recording. The emission fluorescence signal from the fura-2-loaded muscle was filtered through a 510-nm filter and collected by a photomultiplier tube. Background fluorescence from the unloaded muscle was subtracted from the corresponding emission fluorescence signal from the fura-2-loaded muscle strip. The data were recorded using a real-time data-acquisition program (LabVIEW, National Instruments, Austin, TX). To minimize photobleaching, the excitation shutter was only opened during data recording. The emission fluorescence signal from the fura-2-loaded muscle was filtered through a 510-nm filter and collected by a photomultiplier tube. Background fluorescence from the unloaded muscle was subtracted from the corresponding emission fluorescence signal at 340 and 380 nm. 

**Experimental protocol.** All experiments started at baseline temperature (30°C), which kept the progressive fura-2 loss small enough to carry out the 2.5-h experimental protocol (26). Twitch force and [Ca^{2+}], transients were recorded at the following specific steps: baseline temperature before the tissue was cooled (30°C, step 1), initial hypothermia (15°C, 0 h, step 2), hypothermia maintained for 1.5 h (15°C, 1.5 h, step 3), and after the tissue was rewarmed to the baseline temperature (30°C, step 4). The duration of cooling and rewarming the muscle tissue was 30 min. Time-matched controls were maintained at 30°C for 2.5 h. The cooling/rewarming ratio protocol is shown in Fig. 1. Hypothermia at 15°C was chosen to mimic the core body temperature (15°C) of rats used in our previous in vivo rat model of rewarming shock (16, 17, 37).

**Permeabilized papillary muscle fibers.** In a separate set of experiments, LV papillary muscles from the control and rewarming groups were teased into thin strips (~1 mm in length and <200 μm in diameter) in relaxing solution containing the following: 85 mM K+, 5 mM MgATP, 1 mM Mg^{2+}, 7 mM EGTA, and 70 mM imidazole, with a total ionic strength of 150 mM and pH 7.0 with propionic acid (HPr). A computer program was used to calculate the free Ca^{2+} concentration for the activating and relaxing solutions. Dissected thin fibers were permeabilized in relaxing solution containing an additional 1% (vol/vol) Triton X-100 for 1 h at room temperature (20–22°C). Subsequently, the permeabilized fibers were mounted in the Guth Muscle Research System (14) and rinsed for 10 min with relaxing solution. The sarcomere length of the fibers was set to ~2.2 μm using a calibrated monocular microscope (×20 long focal length lens for positioning the whole fiber strip inside the quartz cuvette and ×40 lens for adjusting the sarcomere length, respectively) at 30°C. Varied pCa solutions (6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.5, and 4.0) were made by mixing the activating solution with the relaxing solution on the basis of a computer program-determined ratio (9). [Ca^{2+}] in the cuvette perfusing a permeabilized muscle strip was sequentially varied from pCa 8.0 to 4.0 to obtain force-pCa relationships. Force-pCa relationships were fitted with the following Hill equation:

\[
F = F_{\text{max}} \frac{1}{1 + 10^{nH(pCa - pCa_{50})}}
\]

where F is the steady-state force at various pCa, pCa_{50} is the pCa required for 50% of F_{max}, and n_{H} is the Hill coefficient. pCa_{50} and n_{H} were used as an index of myofibrillar Ca^{2+} sensitivity on the force development and an index of cooperativity of myofibrillar proteins, respectively. Fibers were subjected to a test activation/relaxation cycle (at pCa 4.0 and 8.0, respectively) before the measurement of force-pCa relationships.

**Western blot analysis.** To analyze the phosphorylation of cTnl, a separate set of experiments was performed using LV papillary muscle samples (5–8 mg) from time-matched control andrewarmed groups. All steps were carried out at 4°C to avoid protein degradation. Tissue samples were homogenized in buffer solution containing the following: 20 mM Tris (pH 8.0), 0.15 mM NaCl; 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 10 mM EDTA, and 10 mM sodium azide. Homogenized samples were solubilized in a sample rotator, placed in a cold room for 20 min, and spun in a refrigerated centrifuge at 10,000 g for 10 min. Protein content was assayed by a Lowry assay (Bio-Rad DC protein Assay). Protein (50 μg) was denatured in 1× sample buffer at 100°C for 3 min, fractionated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Transferred proteins on separate PVDF membranes were detected with specific antibodies for either total cTnl [polyclonal antibodies produced with the following epitope: NH2-terminal (102–110) and COOH-terminal (170–180), Cell Signaling, Santa Cruz, CA] or phospho-cTnl at Ser23/24 (Cell Signaling) and visualized by a highly sensitive enhanced chemiluminescent substrate for detecting horseradish peroxidase on immunoblots (Pierce Biotechnology, Rockford, IL). The bands were quantitatively analyzed with molecular imaging software (version 4.0.1, Kodak).

**2-D gel electrophoresis.** To complement the Western blot analysis results, which only showed cTnl phosphorylation at specific Ser23/24 residues, total cTnl phosphorylation in time-matched control andrewarmed groups was determined using 2-D gel electrophoresis (isoelectric point for cTnl: 9.6). The spots were quantitatively analyzed with ImageQuant software (version 5.2, Amersham Bioscience, Piscataway, NJ). The detailed protocols for 2-D gel electrophoresis were previously described (25). In brief, LV papillary muscle samples (5–8 mg) from control andrewarmed groups were extracted with 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% immobilized pH gradient IPG buffer (pH 7–11), and 40 mM DTT. Extracts were treated with a 2-D clean-up kit (Amersham Biosciences), loaded with rehydration buffer solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, and 0.002% bromophenol blue, and then kept in a freezer (−70°C), pH 7–11 IPG dry gel strip (Amersham Biosciences) was laid on rehydration buffer was rehydrated overnight. After rehydration, proteins were isoelectrically focused in a Protein IEF Cell system (Bio-Rad) equipped with a cup loading tray. After isoelectric point separation or isoelectric focusing (IEF), gel strips were equilibrated in 6 M urea, 75 mM Tris·HCl (pH 8.8), 30% glycerol, 2% (wt/vol) SDS, and 0.002% bromophenol blue with 130 mM DTT and 135 mM iodoacetamide before undergoing SDS-PAGE for protein separations by molecular weight. After SDS-PAGE using 12.5% Tris·HCl criterion precast gels (Bio-Rad), the gels were silver stained for the

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**Fig. 1.** Experimental protocol showing the four experimental steps. CTL, control.
analysis of cTnI phosphorylation or transferred to a PVDF membrane for Western blot analysis to identify TnI phosphorylation spots.

Statistical analysis. All results are presented as means ± SE. To statistically analyze the changes in twitch force and [Ca\textsuperscript{2+}], in the time-matched control group and in the hypothermia/rewarming groups within experimental steps, one-way ANOVA was used. When significant differences were observed, Student’s t-test with the Bonferroni correction was used as a post hoc analysis. For the comparison of the time-matched control group versus hypothermia/rewarming groups, unpaired two-tailed Student’s t-test was used. Differences were considered significant at $P < 0.05$.

RESULTS

Time-matched control group maintained at 30°C for 2.5 h. A general decline ($<12\%$ of initial control values) in the peak [Ca\textsuperscript{2+}], transient and peak twitch force was found in time-matched controls at baseline temperature (30°C), but there were no significant differences in the peak [Ca\textsuperscript{2+}], transient and peak twitch force between initial (0 h) and final (2.5 h) control values (Table 1). Furthermore, there were no significant changes in control values of time to peak (TTP) and time to 50% decay (TT50%) of [Ca\textsuperscript{2+}], whereas there were significant decreases in TTP (~20% at 2 and 2.5 h) and TT50% (20% at 0.5 h, 30% at 2 h, and 40% at 2.5 h) of twitch force compared with the initial control values (0 h; Table 1).

Changes in [Ca\textsuperscript{2+}] transients and twitch force after tissues were cooled to 15°C (15°C, 0 h). Compared with values at the baseline temperature (30°C), the following characteristics of both the peak [Ca\textsuperscript{2+}], transient and twitch force were significantly increased: amplitude, by 26% and 72%, respectively; TTP, by 184% and 245%, respectively; and TT50%, by 285% and 277%, respectively (Fig. 2, A and B, and Table 1). These significant increases in the [Ca\textsuperscript{2+}], transient and/or force during tissue cooling to 15°C were comparable with those presented in previous studies (12, 18, 20, 32, 37).

Changes in [Ca\textsuperscript{2+}] transients and twitch force during stable hypothermia for 1.5 h at 15°C. At the end of 1.5 h at 15°C, peak [Ca\textsuperscript{2+}] increased by 16%, whereas peak force remained unchanged, compared with values obtained after tissue was cooled to 15°C (0 h at 15°C). TTP in peak [Ca\textsuperscript{2+}] transients remained unchanged, whereas TTP in force was significantly decreased (6.1%). There were significant reductions in TT50% in both peak [Ca\textsuperscript{2+}] transients and force (6% and 13%, respectively) compared with those at 0 h at 15°C (Fig. 2, A and B, and Table 1).

Changes in [Ca\textsuperscript{2+}] transients and twitch force after tissue was rewarmed to baseline temperature (30°C). After the tissue was rewarmed, the peak [Ca\textsuperscript{2+}] transient was increased by 13%, whereas peak force was reduced by 36%, compared with baseline temperature (30°C). TTP in peak [Ca\textsuperscript{2+}] transients returned to within prehypothermic control values, whereas TTP in force decreased by 20%. TT50% in both peak [Ca\textsuperscript{2+}] transients and force was decreased by 10% and 30%, respectively, compared with baseline (Fig. 2, A and B, and Table 1).

Intact myofilament Ca\textsuperscript{2+} sensitivity. By plotting phase-plane loops (the relationship between twitch force and [Ca\textsuperscript{2+}]), which represent a coarse dynamic index of myofilament Ca\textsuperscript{2+} sensitivity during the relaxation phase of twitch force (3, 8, 31), we observed a rightward shift in the relaxation phase during stable hypothermia (15°C) as well as after rewarining in intact papillary muscle, indicating a decrease in myofilament Ca\textsuperscript{2+} sensitivity due to hypothermia and rewarming (Fig. 3).

Force-pCa relationship in permeabilized muscle fibers. $F_{\text{max}}$ was significantly decreased in rewarmed muscle fibers (32.1 ± 3.2 vs. 45.9 ± 3.9 mN/mm\textsuperscript{2} in controls; Fig. 4A). In Fig. 4B,

Table 1. Amplitude, kinetic parameters, and time-matched control values for peak [Ca\textsuperscript{2+}], transients and twitch force at each experimental step

<table>
<thead>
<tr>
<th>Step 1: baseline (0 h)</th>
<th>[Ca\textsuperscript{2+}], mM</th>
<th>Force, mN/mm\textsuperscript{2}</th>
<th>Time to Peak, ms</th>
<th>Time to 50% Decay, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>812.41 ± 133.82</td>
<td>57.03 ± 3.06</td>
<td>177.19 ± 5.32</td>
<td></td>
</tr>
<tr>
<td>Twist force</td>
<td>925.20 ± 51.65</td>
<td>52.01 ± 2.83</td>
<td>204.50 ± 9.71</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.71 ± 1.29</td>
<td>118.13 ± 7.82</td>
<td>86.04 ± 6.53</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.28 ± 1.30</td>
<td>116.02 ± 6.99</td>
<td>101.80 ± 4.45</td>
<td></td>
</tr>
<tr>
<td>Step 2: initial hypothermia (0.5 h)</td>
<td>1,025.85 ± 192.91*</td>
<td>162.09 ± 8.55*</td>
<td>681.89 ± 30.23*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>910.31 ± 135.25</td>
<td>45.50 ± 1.72</td>
<td>178.50 ± 29.50</td>
<td></td>
</tr>
<tr>
<td>Twist force</td>
<td>13.23 ± 2.22*</td>
<td>407.97 ± 13.36*</td>
<td>324.51 ± 22.62*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.57 ± 0.98</td>
<td>103.40 ± 5.84</td>
<td>81.80 ± 7.22†</td>
<td></td>
</tr>
<tr>
<td>Step 3: maintained hypothermia (2.0 h)</td>
<td>1,190.66 ± 205.17*</td>
<td>166.99 ± 8.48*</td>
<td>625.98 ± 19.30*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>917.24 ± 42.89</td>
<td>50.02 ± 2.84</td>
<td>192.05 ± 15.34</td>
<td></td>
</tr>
<tr>
<td>Twist force</td>
<td>13.43 ± 2.30*</td>
<td>382.51 ± 11.38*</td>
<td>279.00 ± 18.41*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.63 ± 0.75</td>
<td>92.20 ± 4.37†</td>
<td>70.80 ± 3.34†</td>
<td></td>
</tr>
<tr>
<td>Step 4: rewarmed to baseline temperature (2.5 h)</td>
<td>1,937.99 ± 154.89*</td>
<td>56.29 ± 3.07</td>
<td>162.14 ± 4.41*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>815.86 ± 78.49</td>
<td>54.50 ± 9.19</td>
<td>171.50 ± 19.50</td>
<td></td>
</tr>
<tr>
<td>Twist force</td>
<td>4.73 ± 0.68*</td>
<td>96.31 ± 4.46*</td>
<td>62.77 ± 2.97*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.31 ± 0.93</td>
<td>91.40 ± 4.46*</td>
<td>59.60 ± 2.23†</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as means ± SE; $n = 7$ rats for experimental steps and 4 rats for time-matched control at 30°C. [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+} concentration. $*P < 0.05$ vs. baseline temperature (30°C); †$P < 0.05$ vs. initial control values (0 h).

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the data for $F_{\text{max}}$ are normalized to maximal values and plotted against increasing pCa concentrations. In rewarmed muscle fibers, pCa50 was significantly reduced (5.57 ± 0.03 vs. 5.71 ± 0.06 in controls). However, $n_{\text{H}}$ remained unchanged (3.19 ± 0.13 in rewarmed muscle fibers vs. 3.04 ± 0.27 in controls).

**Western blot analysis of cTnI.** The bands of cTnI phosphorylated at Ser23/24 residues (top) as well as total cTnI protein expression (bottom), used as a reference from time-matched control and rewarmed LV papillary muscles, are shown in Fig. 5A. In rewarmed papillary muscle, cTnI phosphorylation was significantly increased compared with that of time-matched control (114.33 ± 7.64 vs. 93.02 ± 3.27 arbitrary units; Fig. 5B).

**2-D gel electrophoresis analysis.** Figure 6A shows typical spots of proteins separated by IEF based on the isoelectric points of specific proteins. Total cTnI phosphorylation in rewarmed papillary muscles was significantly increased compared with that of time-matched controls (51.63 ± 5.19 vs. 33.96 ± 4.69 arbitrary units; Fig. 6B).

**DISCUSSION**

The major findings in the present study are that rewarming after 1.5 h of stable hypothermia increased PKA-mediated TnI phosphorylation as well as the total phosphorylation of cardiac TnI and decreased the Ca$^{2+}$ sensitivity of force in myocardial contractile tissue. These novel data, in conjunction with a substantial reduction in contractile force after rewarming, add to our understanding of the mechanisms underlying hypothermia-induced cardiac contractile dysfunction and also point to a potential therapeutic target to counteract rewarming-induced cardiac failure.

Cooling and rewarming alter myocardial contractile function. After rewarming, papillary muscle twitch force was reduced by ~35% despite a ~15% increase in the [Ca$^{2+}$], transient, indicating that reduced contractile force after rewarming is related to a decrease in the myofilibrillar Ca$^{2+}$ sensitivity of force rather than an impairment in [Ca$^{2+}$] regulation. Significant increases in the temporal parameters of contraction and relaxation in twitch force after rewarming likely reflect a change in myosin/actin cross-bridge cycling kinetics (Table 1).

Despite the fact that in the present study we did not measure cross-bridge rate constants or stiffness, these findings support another explanation for reduced force after hypothermia/rewarming on the basis of a two-state model (5). According to the two-state model, maximum isometric force is defined as follows: maximum isometric force = $nF_{\alpha_{fs}}$, where $n$ is the number of cross-bridges within the half sarcomere, $F$ is the average force per cross-bridge, and $\alpha_{fs}$ is the fraction of cross-bridges in a strongly bound force-generating state. Furthermore, $\alpha_{fs}$ is defined as follows: $\alpha_{fs} = f_{\text{app}}/(f_{\text{app}} + g_{\text{app}})$, where $f_{\text{app}}$ and $g_{\text{app}}$ are the apparent rate constants for cross-bridge attachment and detachment, respectively. Thus, the reduced force development after rewarming may be attributable to the decrease in cross-bridge recruitment ($\alpha_{fs}$) resulting from increased $g_{\text{app}}$ during hypothermia/rewarming. This assumption is supported by our results showing that after tissue rewarming, there were significant decreases in TT50% of the [Ca$^{2+}$] transient as well as twitch force (~10% and 30%, respectively; Table 1).
Decreased myofilament Ca$^{2+}$/H11001sensitivity as an underlying mechanism of posthypothermic contractile dysfunction. Ca$^{2+}$/H11001 activates cardiac muscle in a graded manner, which makes the relationship between [Ca$^{2+}$/H11001]i and force development of functional importance (2). Using intact papillary muscle, we found a significant increase in both the twitch force and peak [Ca$^{2+}$/H11001]i transient at 15°C (Table 1 and Fig. 2, A and B). These findings are comparable with those in previous studies reporting that contractility is greater at 23–25°C than at normothermia in the papillary muscle of various species (20). It is known that temperature change is one of the major factors that alters cardiac myofilament Ca$^{2+}$/H11001 sensitivity (1), but controversy exists whether cardiac myofilament Ca$^{2+}$/H11001 sensitivity will increase or decrease during hypothermia. Stowe et al. (32) reported that in isolated hearts, mild hypothermia (27°C) increases myofilament Ca$^{2+}$/H11001 sensitivity, whereas moderate hypothermia (17°C) decreases it. These findings support our observations that twitch force in intact papillary muscle reaches a maximum at 25°C and then declines as temperature is reduced to 15°C. However, it needs to be emphasized that the significantly increased peak [Ca$^{2+}$/H11001] transient at 15°C in the present experiments (Table 1) may counteract an expected decrease in twitch force due to reduced Ca$^{2+}$/H11001 sensitivity at this temperature. By plotting phase-plane loops (the relationship between twitch force and [Ca$^{2+}$/H11001]), which represent a coarse dynamic index of myofilament Ca$^{2+}$/H11001 sensitivity during the relaxation phase of twitch force (3, 8, 31), we observed a rightward shift in the relaxation phase during stable hypothermia (15°C) as well as after rewarming in intact papillary muscle, indicating a decrease in myofilament Ca$^{2+}$/H11001 sensitivity due to hypothermia and rewarming (Fig. 3). We used skinned papillary muscle fibers to better describe the Ca$^{2+}$/H11001 sensitivity and contractile force after rewarming and found that both Fmax and myofilament Ca$^{2+}$/H11001 sensitivity were significantly decreased in rewarmed cardiac muscle fibers. These data suggest that hypothermia decreases cardiac myofilament Ca$^{2+}$/H11001 sensitivity, which is not reversed by rewarming, thus contributing to posthypothermic cardiac contractile dysfunction.

Increased cTnI phosphorylation and cardiac contractile dysfunction but maintained relaxation characteristics after tissue rewarming. Numerous studies (6, 7, 19, 24, 33, 44) have demonstrated that increased PKA-mediated phosphorylation of the sarcomeric protein cTnI reduces myofilament Ca$^{2+}$/H11001 sensitivity and shifts the force-pCa relationship rightward under normothermic circumstances. To the best of our knowledge, the present study is the first to show that rewarming after stable hypothermia simultaneously increases cTnI phosphorylation and reduces myofilament Ca$^{2+}$/H11001 sensitivity and Fmax. In principle, during systole, Ca$^{2+}$/H11001 moves into the cytoplasmic space and binds to the myofilaments, and contraction starts.
increase the contractile force of the sarcomere is to increase cytosolic Ca\(^{2+}\); another is to regulate how the sarcomeres respond to Ca\(^{2+}\). The unique position of cTnI to regulate and modulate cardiac function under the influence of adrenergic signaling is well documented (30). In more detail, cTnI has a NH\(_2\)-terminal extension of some 30 amino acids that houses Ser23/24, which is phosphorylated by PKA to depress sarcomere sensitivity to Ca\(^{2+}\) and increase cross-bridge kinetics (15). In the present study, we found that after tissues were rewarmed, there was a significant increase in cTnI phosphorylation at PKA sites (Ser23/24), as shown by Western blot analysis (Fig. 5A) and 2-D gel (Fig. 6A), which likely included all phosphorylatable sites on cTnI (Ser23/24, Ser43/45, Thr144, and Ser149). Therefore, we could not exclude other possible phosphorylatable sites on cTnI (Ser43/45, Thr144, and Ser149) that might be phosphorylated by hypothermia/rewarming, even though Ser23 and Ser24 are known to be the dominant phosphorylatable sites on cTnI. Those possible sites on cTnI to be identified in future studies would require the use of the mass spectrometry technique.

Phosphorylation of cTnI is mediated by several kinases [PKA, PKC, PKG, and PAK3 (p21-activated kinases)] (19), but PKA-mediated phosphorylation via β-adrenergic stimulation plays a major physiological role in meeting the increased circulatory demand in the physiological state (19). Interestingly, unlike other pathological clinical conditions, such as ischemia-reperfusion injury, hypertrophy, sepsis, and heart failure (7, 10, 11, 34, 41, 43–45), where a slower relaxation rate is a common finding, a faster relaxation rate after rewarming was observed despite a significant reduction of contractile force in the present experiments. Furthermore, we noticed that in time-matched controls at baseline temperature, TTP and TT50% of twitch force were significantly reduced after 2.5 h (Table 1), whereas TTP and TT50% of [Ca\(^{2+}\)] remained statistically unchanged. Therefore, we speculate that \(f_{app}\) and \(g_{app}\) may be altered in response to the baseline temperature in this time-matched control group.

Yet, it is a challenge to understand the exact mechanism of the direct effects of hypothermia and rewarming on β-adrenergic stimulation leading to PKA-mediated cTnI phosphorylation. In general, changes in the cTnI phosphorylation status, as reported in the failing human heart (7, 10, 11, 34, 41, 43–45), are considered a change in the balance between kinase and phosphatase activities, and the optimal balance is to be found during normal cardiac function. Thus, hypothermia and rewarming, depending on severity and depth, appear to tip the balance between kinase and phosphatase activities, leading to alterations in myofilament Ca\(^{2+}\) sensitivity and myocardial contractility.

Considering the similarities in the changes between cardiac contractile functional variables of in vitro papillary muscle preparations and those of the whole intact heart after experimental hypothermia and rewarming previously studied by our group (16, 17, 36, 38, 39), it is tempting to make a direct comparison between them. However, such a comparison has to be made with great caution. Nevertheless, previous reports (22, 35) have shown that rewarming intact hearts from stable hypothermia (25–15°C) resulted in a significant (~50%) reduction in systolic function but maintained diastolic cardiac function. Thus, similarities in cardiac contractile functional variables between in vitro papillary muscle and the intact heart may be ascribed to a hypothermia-induced decrease in Ca\(^{2+}\) sensitivity that persists after rewarming. PKA-dependent phosphorylation of cTnI, induced by hypothermia and rewarming, may explain both the reduction in systolic functional characteristics and proper maintenance in diastolic function in the rewarmed intact heart. Furthermore, we (16) have reported a lack of effect of pharmacological agents to support cardiac contractile function mediated via the β-receptor-cAMP-PKA pathway during as well as after rewarming. Likewise, if hypothermia and rewarming per se activate the β-receptor-cAMP-PKA pathway, one may interpret that an additional pharmacological stimulus under these circumstances of the β-receptor would bring about much less of cardiac inotropic effect. As already outlined, these assumptions cannot be applied to draw any conclusions but may be used as a plausible rationale for future research protocols.

Although rewarming shock is considered a variant of irreversible circulatory shock, cardiac contractile dysfunction after rewarming may range from cardiac stunning to severe acute cardiac failure. The present findings may also have relevance to rewarming hypothermic patients after heart surgery as well as to therapeutic hypothermia used to treat patients after being resuscitated after acute cardiac standstill. However, there are still limitations in extrapolating experimental results using...
small animals to human medicine, and we also have an experimental limitation in the present experiments: rewarminng of the muscle fibers before the initiation of the experimental protocol, although care was taken to minimize this additional rewarminng. The isolation of the papillary muscle from the LV, the dye loading, and the muscle skinning preparation were all performed at 22°C, whereas the baseline temperature was 30°C. This temperature of 22°C was chosen due to the fact that fura-2 AM loading into muscle fibers is optimal at this temperature, minimizing the compartmentalization that is usually pronounced at higher loading temperatures.

Conclusions. After rewarminng, myocardial contractility is significantly reduced, as determined by the reduced twitch force in LV intact papillary muscle and also by the decreased myofibrillar Ca\(^{2+}\) sensitivity and \(F_{\text{max}}\) in permeabilized muscle fibers. The reductions in twitch force, myofibrillar Ca\(^{2+}\) sensitivity, and \(F_{\text{max}}\) likely result from increased cTnI phosphorylation in the present model. An increase in cTnI phosphorylation possibly involves not only PKA but also other kinases activated by hypothermia and rewarminng.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

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