Protein kinase A–dependent modulation of Ca^{2+} sensitivity in cardiac and fast skeletal muscles after reconstitution with cardiac troponin

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Protein kinase A (PKA)-dependent phosphorylation of troponin (Tn)I represents a major physiological mechanism during β-adrenergic stimulation in myocardium for the reduction of myofibrillar Ca^{2+} sensitivity via weakening of the interaction with TnC. By taking advantage of thin filament reconstitution, we directly investigated whether or not PKA-dependent phosphorylation of cardiac TnI (cTnI) decreases Ca^{2+} sensitivity in different types of muscle: cardiac (porcine ventricular) and fast skeletal (rabbit psoas) muscles. PKA enhanced phosphorylation of cTnI at Ser23/24 in skinned cardiac muscle and decreased Ca^{2+} sensitivity, of which the effects were confirmed after reconstitution with the cardiac Tn complex (cTn) or the hybrid Tn complex (designated as PCRF; fast skeletal TnT with cTnI and cTnC). Reconstitution of cardiac muscle with the fast skeletal Tn complex (sTn) not only increased Ca^{2+} sensitivity, but also abolished the Ca^{2+}-desensitizing effect of PKA, supporting the view that the phosphorylation of cTnI, but not that of other myofibrillar proteins, such as myosin-binding protein C, primarily underlies the PKA-induced Ca^{2+} desensitization in cardiac muscle. Reconstitution of fast skeletal muscle with cTn decreased Ca^{2+} sensitivity, and PKA further decreased Ca^{2+} sensitivity, which was almost completely restored to the original level upon subsequent reconstitution with sTn. The essentially same result was obtained when fast skeletal muscle was reconstituted with PCRF. It is therefore suggested that the PKA-dependent phosphorylation or dephosphorylation of cTnI universally modulates Ca^{2+} sensitivity associated with cTnC in the striated muscle sarcomere, independent of the TnT isoform.

INTRODUCTION

In the mammalian heart, β-adrenergic stimulation activates PKA coupled with a rise of cAMP, resulting in phosphorylation of several membrane- and sarcomere-based proteins in the cardiomyocyte (for reviews with original citations see Bers, 2001, 2002). Accordingly, myocardial function is associated with increased developed force and increased rates of rise and fall of developed force. It is generally considered that changes in the intracellular Ca^{2+} concentration are primarily responsible for these effects (Kurihara and Konishi, 1987; Endoh and Blinks, 1988; Li et al., 2000; Bers, 2001, 2002). In the sarcomere, PKA principally targets the thin filament protein troponin (Tn)I, the thick filament protein myosin-binding protein C (MyBP-C) (e.g., Bers, 2001, 2002), and the giant

elastic protein titin (connectin) (Yamasaki et al., 2002; Fukuda et al., 2005, 2008). Phosphorylation of cardiac TnI (cTnI) and MyBP-C has been reported to decrease myofibrillar Ca^{2+} sensitivity and to enhance cross-bridge cycling, which likely contributes to the increased rate of relaxation and decreased twitch duration (Hoh et al., 1988; Hongo et al., 1993; Kentish et al., 2001). Diastolic properties are also affected during β-adrenergic stimulation, with a reduction in myocardial stiffness due to titin phosphorylation at the N2B segment in the I-band (both N2B and N2BA titins: Yamasaki et al., 2002; Fukuda et al., 2005, 2008).

Because cardiac myofilaments are activated by Ca^{2+} in a graded fashion, in the range of the submaximal Ca^{2+} concentration, the relationship between the Ca^{2+} concentration and active force is of fundamental importance (Bers, 2001, 2002). In this process, Tn switches the thin filament state to "off" or "on" (two-state model; e.g., Solaro and Rarick, 1998), or to "blocked, "closed," or "open" (three-state model; e.g., Gordon et al. 2000). Tn is a heterotrimer of the following distinct gene products:

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i.e., TnC (a Ca^{2+} receptor), TnI (an inhibitor of actomyosin interaction), and TnT (an anchor that binds to tropomyosin [Tm]) (e.g., Solaro and Rarick, 1998; Ohtsuki and Morimoto, 2008). The cardiac isoform of TnI has a far N-terminal extension consisting of $\sim$30 amino acids, which is unique to this molecule (e.g., Solaro and Rarick, 1998). It is well documented that two Ser residues (Ser23 and Ser24) in the N-terminal extension are phosphorylated by PKA, resulting in enhanced relaxation via a decrease in myofibrillar Ca^{2+} sensitivity (for review see Solaro and Rarick, 1998; Layland et al., 2005). Recently, Howarth et al. (2007) demonstrated a clear conformational transition in the cardiac Tn complex (cTn) upon Ser23/24 phosphorylation in cTnI. They proposed that Ser23/24 phosphorylation extends and stabilizes the C-terminal helix in the N-terminal extension of cTnI, and weakens interactions with the N-lobe of cTnC. This may result in bending of cTnI and positioning of the acidic N terminus for electrostatic interactions with the electrostatic inhibitory region of cTnI. Skeletal TnI, from either fast or slow muscle, does not possess PKA phosphorylation sites in the N-terminal region and, therefore, does not exhibit a decrease in Ca^{2+} sensitivity upon PKA treatment (e.g., Solaro and Rarick, 1998). Indeed, it has been reported that cardiac muscle from a mouse model expressing slow skeletal TnI in the heart does not exhibit a decrease in Ca^{2+} sensitivity when exposed to β-adrenergic stimulation (Fentzke et al., 1999). More recent studies using mutant forms of cTnI with either pseudo-phosphorylated PKA sites (cTnI-S23D, S24D) or nonphosphorylatable mutant cTnI-S23A, S24A strongly support a critical role of cTnI in cardiac contractility during β-adrenergic stimulation (for review see Solaro et al., 2008).

Here, we tested whether the PKA-dependent control of phosphorylation or dephosphorylation of cTnI modulates Ca^{2+} sensitivity in different types of muscle by using our Tn reconstitution technique (Terui et al., 2008). We found that PKA decreases Ca^{2+} sensitivity not only in cardiac muscle, but also in fast skeletal muscle after thin filaments had been reconstituted with cTn or the hybrid Tn complex (PCRF) consisting of cTnI and cTnC and fast skeletal TnT (sTnT). Our findings demonstrate a universal role of cTnI via PKA activation in the modulation of Ca^{2+} sensitivity associated with cTnC in the striated muscle sarcomere.

MATERIALS AND METHODS

All experiments performed in this study conform to the Guide for the Care and Use of Laboratory Animals (1996. National Academy of Sciences, Washington D.C.).

Preparation of skinned muscles
Skinned cardiac muscles were prepared based on our recent article (Terui et al., 2008). In brief, porcine hearts (animals, $\sim$1.0 years old) were obtained from a local slaughterhouse. Muscle strips (1–2 mm in diameter and $\sim$10 mm in length) were dissected from the papillary muscle of the left ventricle in Ca^{2+}-free Tyrode's solution (see Terui et al., 2008 for composition containing 30 mM 2,3-butanedione monoxime [BDM] to inhibit actomyosin interaction), with 10 μM propranolol to block nonspecific β-adrenergic stimulation and 10 μM carbamoylcholine to enhance phosphatase activity (Gupta et al., 1994). BDM is also known to enhance dephosphorylation of myofibrillar proteins (Venema et al., 1993; Turnbull et al., 2002), including cTnI and MyBP-C. The presence of propranolol, carbamoylcholine, and BDM, however, does not exclude basal levels of protein phosphorylation (Fukuda et al., 2005 and this study). Psoas muscles were obtained from white rabbits (2–3 kg). The muscle strips were skinned in relaxing solution (5 mM MgATP, 40 mM N,N-bis (2-hydroxy-ethyl)-2-aminoethanesulfonic acid [BES], 1 mM Mg^{2+}, 10 mM EGTA, 1 mM dithiothreitol, 15 mM phosphocreatine, 15 U/ml creatine phosphokinase, and 180 mM ionic strength [adjusted by K-propionate], pH 7.0) containing 1% (wt/vol) Triton X-100 and 10 mM BDM overnight at $\sim$3°C. They were stored for up to 3 wk at -20°C in relaxing solution containing 50% (vol/vol) glycerol. All solutions contained protease inhibitors (0.5 mM PMSF, 0.04 mM leupeptin, and 0.01 mM E64) to avoid protein degradations (see Fukuda et al., 2003).

Skinned muscle mechanics
Isometric force was measured at 15°C based on our previous reports with either thin cardiac bundles (porcine left ventricular [PLV]; $\sim$100 μm in diameter and $\sim$2 mm in length; see Terui et al., 2008) or single skeletal fibers (rabbit psoas [RP]; see Udaka et al., 2008). In brief, sarcomere length (SL) was measured by laser diffraction during relaxation (as in Fukuda et al., 2000, 2001a,b) and set at 2.0 μm for both PLV and RP. To avoid the possible effects of PKA-dependent passive force modulation on active force, we conducted all experiments at SL 2.0 μm, where passive force is barely produced in both PLV and RP (e.g., Cazorla et al., 2000; Prado et al., 2005; Terui et al., 2008). Just before contraction, the preparation was immersed in low-EGTA (0.5 mM) relaxing solution to avoid slowing of contraction (Fukuda et al., 2001a). The preparation was first activated at pCa 4.5 to obtain maximal Ca^{2+}-activated force, followed by relaxation. The preparation was then activated at various pCa's (from high to low pCa, and lastly at pCa 4.5) to construct the force-pCa curve (i.e., force-pCa protocol). The curves were fitted to the Hill equation, and the value of the midpoint of the force-pCa curve (i.e., pCa_{50}) was used as an index of myofibrillar Ca^{2+} sensitivity (Fukuda et al., 2000). The Hill coefficient (n_H) was used as an index of thin filament cooperative activation (Fukuda et al., 2000). Here, forces at submaximal Ca^{2+} levels were normalized to the maximal force obtained at the end of the force-pCa protocol (rundown in active force during the protocol was <10%). With the values of pCa_{50}, n_H, and maximal force being similar in the presence of a higher concentration of creatine phosphokinase (225 U/ml; not depicted), we used 15 U/ml throughout the study for both PLV and RP.

Next, the preparation was incubated for 50 min at 22°C with purified PKA (catalytic subunit from bovine heart; Sigma-Aldrich) at a concentration of 1 U/μl (Fukuda et al., 2005). PKA-specific inhibitor (PKI; Sigma-Aldrich) was used in control experiments at 50 μM (Fukuda et al., 2005). Then, the force-pCa protocol was repeated and the force-pCa curve was obtained. The difference between the values of pCa_{50} (ΔpCa_{50}) before and after PKA treatment was used as an index of the PKA-induced Ca^{2+} desensitization (PKA effect).

To reduce the background phosphorylation of myofibrillar proteins, we used protein phosphatase 1 (PP1; Sigma-Aldrich) (see Jideama et al., 2006). After conducting the force-pCa protocol, cardiac muscle preparations were immersed in relaxing solution containing PP1 at 900 U/ml for 1 h at 25°C. Then, the

preparations were immersed in normal relaxing solution at 15°C for 10 min with gentle agitation to remove PP1 for subsequent PKA treatment.

Tn extraction
The fast skeletal Tn complex (sTn) was purified from rabbit fast skeletal muscle based on our previously published procedure (Shiraishi et al., 1993) and stored at −80°C.

Porcine ventricular and rabbit fast skeletal Tn subunits were prepared by the method of Ojima and Nishita (1986), with slight modifications. In brief, Tn components were separated by the two-step ion-exchange chromatography in the presence of 6 M urea and 1 mM EDTA, with Toyopearl DEAE-650M and CM-650M (Tosoh) columns (2.0 cm i.d. × 20 cm) (see Ojima and Nishita, 1988 for details). To reconstitute the cardiac Tn and sTnT-substituted Tn, the three Tn components (60 µM final of each) were mixed at a 1:1:1 molar ratio and dialyzed as follows: (1) The mixtures were dialyzed for 3 h against 6 M urea, 0.5 M KCl, 10 mM Tris-HCl, pH 7.6, and 5 mM 2-mercaptoethanol. (2) The concentration of urea in the outer dialysate was decreased stepwise to 3 and 1 M. (3) The urea was removed by dialysis for 6 h against 0.5 M KCl, 10 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01% NaN_3, and 5 mM 2-mercaptoethanol. (4) The KCl concentration of the outer dialysate was decreased stepwise to 0.25 and 0.15 M. The precipitate formed was removed by centrifugation (10,000 g, 15 min). The reconstituted Tn complexes were concentrated by centrifugal ultrafiltration with an Apollo concentrator (Orbital Biosciences) and finally dialyzed against 1 mM $NaHCO_3$ and 5 mM 2-mercaptoethanol. Reconstituted Tn complexes were stored at −80°C.

Tn exchange
Tn exchange was performed based on our previously published procedure (Terui et al., 2008). In brief, after performing the force-pCa protocol without any treatment (as described above), the skinned muscle preparation, either PLV or RP, was bathed in rigor solution (10 mM BES, 150 mM K-propionate, 2.5 mM EGTA, and 5 mM $MgCl_2$, pH 7.0) containing cTn or PCRF (2 and 6 mg/ml for PLV and RP, respectively) and 80 mM BDM for 60 min at 25°C. sTn reconstitution was performed on PLV at 2 mg/ml for 20, 40, or 60 min at 25°C (60 min for quasi-complete reconstitution; see Terui et al., 2008). The time-dependent change in cTnI upon sTn reconstitution was assessed using the ratio cTnI/(cTnI + sTnI). As noted in our previous report (Terui et al., 2008), the presence of BDM was essential to avoid contraction upon exchange and the ensuing damage to the sarcomere structure. Next, the preparation was washed with normal relaxing solution at 15°C for 10 min with gentle agitation to remove excess Tn molecules. In experiments with cTn- or PCRF-reconstituted RP, the fibers were further treated with sTn at 2 mg/ml for 60 min at 25°C after PKA treatment.

Electrophoresis
SDS-PAGE was performed based on our previous studies (Terui et al., 2008; Udaka et al., 2008). Muscle preparations were solubilized and electrophoresed on Laemmli gels (acrylamide, 3.5% in stack and 15% resolving gel, pH 9.3; stained with Coomassie brilliant blue). Tn isoforms were identified by commigration with known standards, as performed in our previous study (Terui et al., 2008). We quantified the reconstitution ratio by comparing the ratio of TnI or TnT to actin with that obtained for the original muscle (i.e., either RP or PLV muscle). Due to the technical difficulty in the quantitative analysis of TnC, especially in RP where cTnC and sTnC coexist after reconstitution (with only a slight difference in molecular weight), we present only the analyzed data on TnT and TnI, based on our previous study (Terui et al., 2008).

Western blotting
Immunoblotting was performed based on our previous report (O-Uchi et al., 2008). After separation on 20% SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane. For the detection of PKA-dependent Ser23/24 phosphorylation in cTnI (pig cTnI; GenBank accession no. DQ534449.1), the transferred membranes were blocked by Blocking One-P solution (Nakalai Tesque, Inc.), which is free of phosphate groups and endogenous phosphates, and incubated with cardiac phospho-TnI (Ser23/24) polyclonal antibody (Cell Signaling Technology). Then, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (GE Healthcare), and the bands were visualized by using an ECL-plus detection kit (GE Healthcare). Sequentially, after gently stripping these primary and secondary antibodies to allow reprobings on the same membrane, the membranes were blocked by Tris-based buffer containing 5% nonfat skim milk (BD) and 0.1% tween-20. For the detection of total cTnI, we used anti-cTnI monoclonal antibody (Millipore) and secondary horseradish peroxidase–conjugated antibody (GE Healthcare). Radiographs were scanned and digitized (ES-2200; Epson), and densitometry was performed using One-D-Scan (v. 2.03; BD).

Statistics
Significant differences were assigned using the paired or unpaired Student's t test as appropriate. Data are expressed as mean ± SEM, with "n" representing the number of muscles. Statistical significance was assumed to be P < 0.05. "N.S." indicates P > 0.05.

Online supplemental material
Table S1 shows the reproducibility of the values of pCa_{50}, n_H, and maximal force after repeated measurements of the force-pCa curve in PLV and RP under the control condition with no treatment. Fig. S1 shows that (1) PKA phosphorylates MyBP-C in PLV, and (2) cTnI is the only protein among various Tn subunits that shows phosphorylation upon PKA treatment. Table S1 and Fig. S1 are available at http://www.jgp.org/cgi/content/full/jgp.200910206/DC1.

RESULTS

Effect of PKA on Ca^{2+} sensitivity in PLV muscle with and without sTn reconstitution
First, we tested how PKA-dependent phosphorylation of cTnI modulates Ca^{2+} sensitivity in PLV under the control condition without Tn reconstitution. We found that our PKA treatment nearly doubled Ser23/24 phosphorylation in cTnI (Fig. 1 A). Fig. 1 B shows the effect of PKA on myofibrillar Ca^{2+} sensitivity. Consistent with a previous study on porcine ventricular preparations (Zhang et al., 1995), the force-pCa curve was shifted to the right by 0.10 ± 0.01 pCa units (as indexed by pCa_{50}), exhibiting a decrease in Ca^{2+} sensitivity. PKA did not affect maximal force or thin filament cooperative activation (as indexed by n_H; Table I). Ca^{2+} desensitization was not observed when the preparation was treated with PKA in the presence of the PKI (pCa_{50}: 5.57 ± 0.02 and 5.54 ± 0.03, respectively, before and after PKA+PKI; P > 0.05; $n = 6$).

Next, we tested the effect of PKA on Ca^{2+} sensitivity in PLV after reconstitution with sTn (Fig. 1 C). Quasi-complete reconstitution with sTn (for 60 min) increased Ca^{2+} sensitivity by 0.31 ± 0.03 pCa units (see
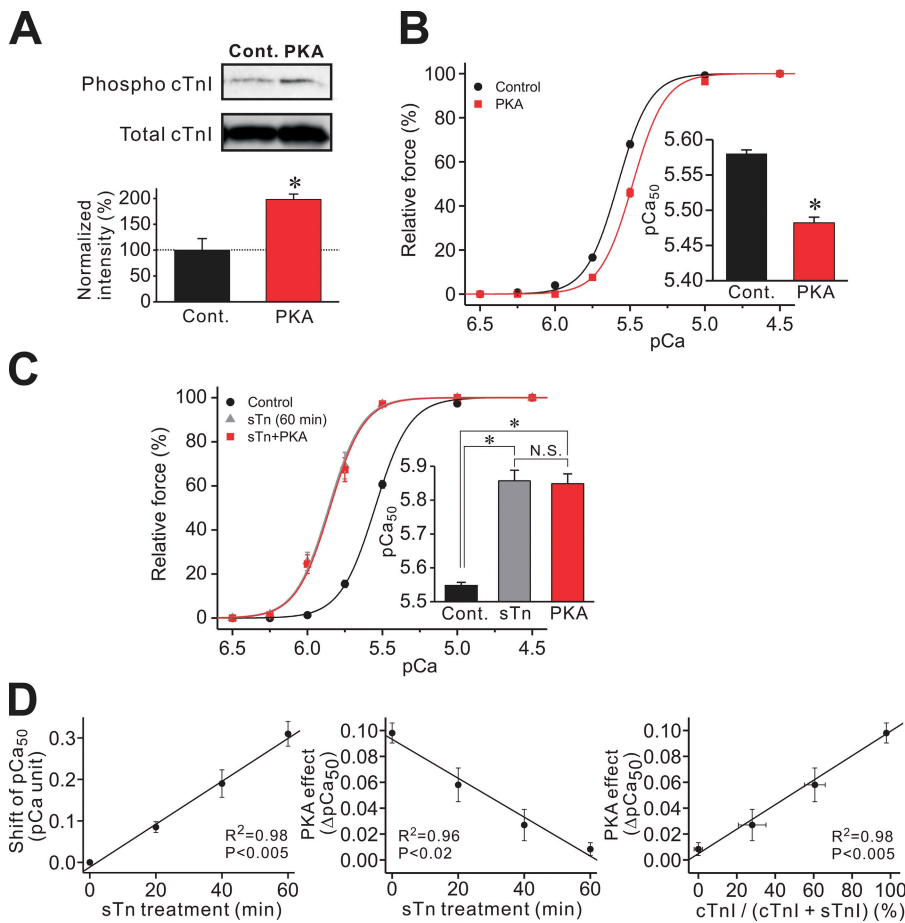
Figure 1. Effects of PKA on cTnI phosphorylation and Ca^{2+} sensitivity in PLV. (A; Top) Western blotting showing cTnI phosphorylation (12.5 µg protein/well). Cont., control without PKA treatment. (Bottom) Comparison of the intensity of cTnI Ser23/24 phosphorylation normalized with total cTnI content in control and PKA-treated PLV. *, $P < 0.05$. $n = 3$. (B) Force-pCa curves showing the effect of PKA on Ca^{2+} sensitivity. (Inset) pCa_{50} values before and after PKA treatment. *, $P < 0.05$. $n = 7$. (C) Force-pCa curves showing the effects of quasi-complete sTn reconstitution (shown in gray) and subsequent treatment with PKA on Ca^{2+} sensitivity. (Inset) pCa_{50} values. *, $P < 0.05$. $n = 7$. (D; Left) Plot of sTn treatment time versus sTn-induced shift of pCa_{50}. (Middle) Plot of sTn treatment time versus PKA-induced shift of pCa_{50}. (Right) Plot of residual cTnI ratio (i.e., cTnI/(cTnI + sTnI)) versus PKA-induced shift of pCa_{50}. $n = 7$.

Terui et al., 2008) and completely abolished the PKA-induced Ca^{2+} desensitization. Increasing the time for sTn reconstitution (up to 60 min) increased Ca^{2+} sensitivity (Fig. 1 D, left) and diminished the PKA effect (Fig. 1 D, middle), both in a linear fashion. A linear relationship was found between the residual amount of cTnI and the PKA effect (Fig. 1 D, right).

TABLE I
Summary of the values of maximal active force and n_H before and after PKA treatment in PLV

	Maximal force (mN/mm²)	n_H
Fig. 1 B		
Control PLV	52.89 ± 3.24	4.06 ± 0.20
PKA	52.23 ± 3.70	4.02 ± 0.22
Control PLV	51.89 ± 3.16	3.98 ± 0.21
PKA + PKI	50.23 ± 3.78	3.97 ± 0.18
Fig. 1 C		
Control PLV	52.06 ± 3.77	3.74 ± 0.24
sTn	47.71 ± 4.59	3.87 ± 0.09
sTn + PKA	46.92 ± 4.85	3.84 ± 0.12

Maximal force was obtained by activating muscle at pCa 4.5 before plotting of the force-pCa curve at each condition. No significant difference was observed in each case.

Effect of PKA on Ca^{2+} sensitivity in PLV muscle reconstituted with cTn or PCRF

Next, we tested whether or not PKA exerts its Ca^{2+}-desensitizing effect in PLV after thin filament reconstitution with cTn or PCRF. The representative 15% gel showed that the band for cTnT or cTnI was unaffected by cTn reconstitution (cTn reconstitution ratio: cTnI, 94.21 ± 9.63%; cTnT, 93.22 ± 5.33%; $n = 6$) (Fig. 2 A; see Terui et al., 2008). Upon PCRF reconstitution, endogenous cTnT was effectively replaced by sTnT, with cTnI appearing similarly as in control PLV (PCRF reconstitution ratio: cTnI, 86.79 ± 8.81%; sTnT, 130.28 ± 4.09%; residual cTnT, 4.91 ± 1.62%; $n = 6$) (Fig. 2 A). Western blotting demonstrated that cTnI Ser23/24 phosphorylation was clearly less in cTn- or PCRF-reconstituted PLV than in control PLV, and that PKA treatment increased the phosphorylation level in both (Fig. 2 B; see legend for quantitative analysis).

As with sTn reconstitution (Table I; see also Terui et al., 2008), maximal force was decreased by a small magnitude (∼5%) upon cTn or PCRF reconstitution in PLV (Table II). However, the decrease was not statistically significant and, therefore, we consider that the damage, if at all, to the sarcomere structure (or the regulatory system) was minimal in our protocol, as reported in our earlier study (Terui et al., 2008).
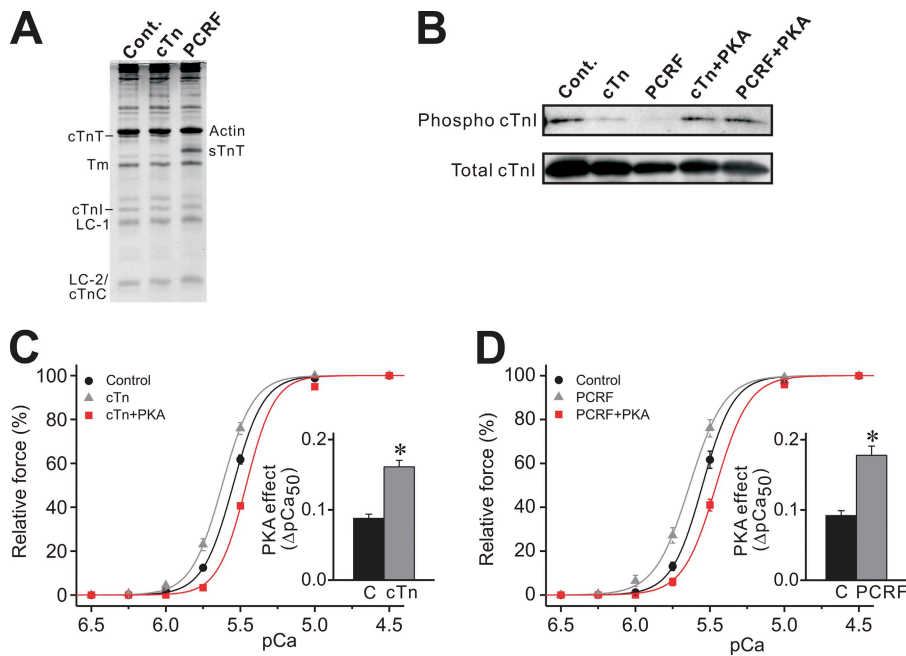
Figure 2. Effects of PKA on cTnI phosphorylation and Ca^{2+} sensitivity in PLV reconstituted with cTn or PCRF. (A; Left) SDS-PAGE for control, cTn-, and PCRF-reconstituted PLV. Cont., control with no reconstitution; cTn, cTn reconstitution; PCRF, PCRF reconstitution; Tm, tropomyosin; LC-1, myosin light chain 1; LC-2, myosin light chain 2. (B) Western blotting showing the effect of PKA on cTnI Ser23/24 phosphorylation in cTn- and PCRF-reconstituted PLV (12.5 µg protein/well). Cont., control with no treatment. Normalized intensity compared with control ($n = 3$): cTn, 10.12 ± 6.53%; PCRF, 13.83 ± 5.23%; cTn + PKA (PKA phosphorylation after cTn reconstitution), 214.94 ± 30.82% (P < 0.05 compared with cTn); PCRF + PKA (PKA phosphorylation after PCRF reconstitution), 228.89 ± 20.13% (P < 0.05 compared with PCRF). (C) Force-pCa curves showing the effects of cTn reconstitution and subsequent PKA treatment on Ca^{2+} sensitivity. (Inset) Difference between the values of the PKA-induced shift of pCa_{50} (C, control minus cTn + PKA; cTn, cTn minus cTn + PKA). *, P < 0.05. $n = 7$. (D) Force-pCa curves showing the effects of PCRF reconstitution and subsequent PKA treatment on Ca^{2+} sensitivity. (Inset) Difference between the values of the PKA-induced shift of pCa_{50} (C, control minus PCRF + PKA; PCRF, PCRF minus PCRF + PKA). *, P < 0.05. $n = 7$. PKA treatment did not significantly affect maximal force or n_H in both C and D (see Table II).

Ca^{2+} sensitivity was slightly but significantly (0.07 ± 0.01 pCa units) increased upon cTn reconstitution, and it was decreased by 0.16 ± 0.01 pCa units after PKA treatment (Fig. 2 C). Similarly, Ca^{2+} sensitivity was increased

by 0.09 ± 0.01 pCa units upon PCRF reconstitution, and it was decreased by 0.18 ± 0.01 pCa units after PKA treatment (Fig. 2 D). Accordingly, the PKA effect was more pronounced with exogenous cTnI (compare with Fig. 1 B). Reconstitution with cTn or PCRF did not significantly affect the maximal force or n_H (Table II).

Effect of PKA on Ca^{2+} sensitivity in PP1-treated PLV muscle
The apparently greater effect of PKA on Ca^{2+} sensitivity in cTn- or PCRF-reconstituted PLV (Fig. 2, C and D) may be coupled with reconstitution of the dephosphorylated form of cTnI and, therefore, a greater magnitude of PKA-dependent phosphorylation of it. We therefore tested the effect of PKA on Ca^{2+} sensitivity in PLV that had been pretreated with PP1 to reduce background phosphorylation of cTnI. Consistent with a previous report (Jideama et al., 2006), PP1 effectively reduced background cTnI Ser23/24 phosphorylation, and the subsequent PKA treatment increased the phosphorylation level (Fig. 3 A; see legend for quantitative analysis).

Ca^{2+} sensitivity was slightly but significantly (0.09 ± 0.01 pCa units) increased upon PP1 treatment, with no significant change in maximal force or n_H (Table II), and it was decreased by 0.18 ± 0.01 pCa units after PKA treatment. Fig. 3 C compares the magnitude of Ca^{2+} sensitization upon PP1 treatment, cTn, and PCRF reconstitution, and the subsequent PKA effect obtained in these preparations. All maneuvers similarly increased

TABLE II
Summary of the values of maximal active force, pCa_{50} and n_H before and after PKA treatment in PLV after reconstitution with cTn or PCRF or treatment with PP1

	Maximal force (mN/mm²)	pCa_{50}	n_H
Fig. 2 C			
Control PLV	55.99 ± 5.92	5.55 ± 0.01	4.23 ± 0.25
cTn	52.52 ± 5.48	5.62 ± 0.01[a]	4.15 ± 0.35
cTn+PKA	51.17 ± 5.42	5.46 ± 0.01[a,b]	4.56 ± 0.30
Fig. 2 D			
Control PLV	53.85 ± 6.03	5.55 ± 0.02	4.16 ± 0.16
PCRF	51.42 ± 5.35	5.64 ± 0.03[a]	3.69 ± 0.16
PCRF + PKA	49.86 ± 5.32	5.46 ± 0.01[a,b]	3.92 ± 0.18
Fig. 3 B			
Control PLV	59.32 ± 4.51	5.55 ± 0.01	4.12 ± 0.16
PP1	56.47 ± 5.70	5.64 ± 0.02[a]	4.24 ± 0.35
PP1 + PKA	54.10 ± 5.66	5.46 ± 0.01[a,b]	4.05 ± 0.20

Maximal force was obtained by activating muscle at pCa 4.5 before plotting of the force-pCa curve at each condition.
[a]P < 0.05 compared with the corresponding values for control PLV (before thin filament reconstitution or PP1 treatment).
[b]P < 0.05 compared with the corresponding values for thin filament reconstitution or PP1 treatment (before PKA treatment).
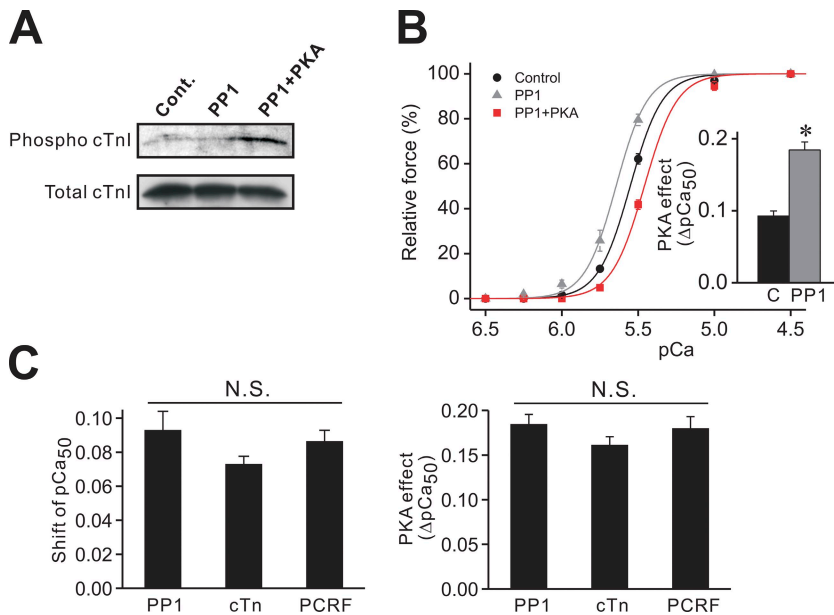
Figure 3. Effects of PKA on cTnI phosphorylation and Ca^{2+} sensitivity in PP1-treated PLV. (A) Western blotting showing the effect of PP1 or PKA on the phosphorylation level of Ser23/24 in cTnI (12.5 μg protein/well). Cont., control with no treatment. Normalized intensity compared with control ($n = 5$): PP1, 40.16 ± 10.48%; PP1 + PKA (PKA phosphorylation after PP1 treatment), 207.76 ± 53.85% ($P < 0.05$ compared with PP1). (B) Force-pCa curves showing the effects of PP1 and subsequent PKA treatment on Ca^{2+} sensitivity. (Inset) Difference between the values of the PKA-induced shift of pCa_{50} (C, control minus PP1 + PKA; PP1, PP1 minus PP1 + PKA). *, $P < 0.05$. $n = 7$. PKA treatment did not significantly affect maximal force or n_H (see Table II). (C; Left) Comparison of Ca^{2+} sensitization as indexed by the shift of pCa_{50} after PP1 treatment and cTn or PCRF reconstitution in PLV. (Right) Comparison of the PKA-induced shift of pCa_{50} in PP1-treated and cTn- or PCRF-reconstituted PLV. Statistical significance was not observed between groups in either graph.

Ca^{2+} sensitivity (Fig. 3 C, left; $P > 0.05$), and the subsequent PKA effect was similar (Fig. 3 C, right; $P > 0.05$).

Effect of PKA on Ca^{2+} sensitivity in cTn-reconstituted RP muscle

We next investigated whether PKA exerts its Ca^{2+}-desensitizing effect in RP after cTn reconstitution (reconstitution ratio, ∼50%; see Terui et al., 2008). As shown in the chart recording (Fig. 4 A), submaximal forces (at pCa 6.0 and 5.75) were decreased upon cTn reconstitution by a magnitude greater than that of maximal force, resulting in a decrease in the percentage to the maximum (see Terui et al., 2008). PKA treatment further decreased submaximal forces with little or no change in maximal force. Lastly, sTn reconstitution restored submaximal forces to levels similar to those obtained before cTn reconstitution, accompanied by a slight increase in maximal force.

Force-pCa curves in Fig. 4 B demonstrate that Ca^{2+} sensitivity is decreased by 0.10 ± 0.01 pCa units with cTn reconstitution (see Terui et al., 2008) and is further decreased by 0.10 ± 0.01 pCa units with PKA treatment. As evident in the chart recording (Fig. 4 A), sTn reconstitution almost completely restored Ca^{2+} sensitivity to the original level before cTn reconstitution. In a control experiment without cTn reconstitution, PKA treatment did not affect Ca^{2+} sensitivity in RP (Fig. 4 C).

Maximal force and n_H were decreased by cTn reconstitution (Terui et al., 2008), but not by PKA treatment, and both were restored with sTn reconstitution to levels not significantly different than those obtained before cTn reconstitution (Table III).

Effect of PKA on Ca^{2+} sensitivity in PCRF-reconstituted RP muscle

Finally, by using PCRF reconstitution, we investigated whether the PKA-induced decrease in Ca^{2+} sensitivity in cTn-reconstituted RP is coupled with the phosphorylation of cTnI or, alternatively, cTnT. As shown in Fig. 5 A, our protocol with PCRF allowed for ∼50% replacement of sTnI with cTnI (PCRF reconstitution ratio: cTnI, 47.80 ± 2.54%; sTnT, 128.52 ± 8.80%; residual sTnI, 43.52 ± 6.51%; $n = 6$). Subsequent treatment with sTn

TABLE III

Summary of the values of maximal active force and n_H before and after PKA treatment in RP after reconstitution with cTn or PCRF

	Maximal force (mN/mm²)	n_H
Fig. 4 B		
Control RP	179.71 ± 7.04	4.47 ± 0.15
cTn	157.86 ± 4.30[a]	3.00 ± 0.12[a]
cTn + PKA	157.57 ± 4.22[a]	3.07 ± 0.15[a]
sTn	171.86 ± 5.18[b,c]	4.71 ± 0.36[b,c]
Fig. 5 C		
Control RP	182.14 ± 7.98	4.50 ± 0.17
PCRF	158.14 ± 5.84[a]	3.20 ± 0.25[a]
PCRF + PKA	157.03 ± 5.71[a]	3.13 ± 0.23[a]
sTn	174.43 ± 5.05[b,c]	4.42 ± 0.16[b,c]

Maximal force was obtained by activating muscle at pCa 4.5 before plotting of the force-pCa curve at each condition.

[a]$P < 0.05$ compared with the corresponding values for control RP (before thin filament reconstitution).

[b]$P < 0.05$ compared with the corresponding values for thin filament reconstitution (before PKA treatment).

[c]$P < 0.05$ compared with the corresponding values for PKA treatment (before sTn reconstitution).
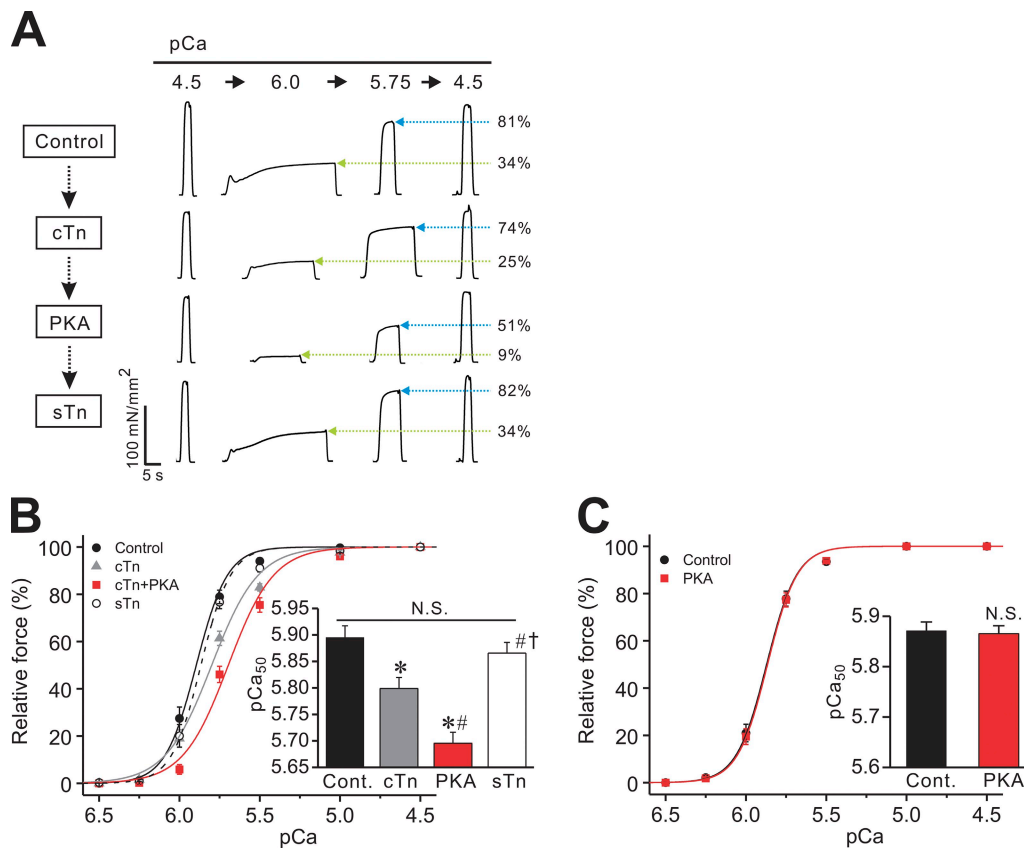
Figure 4. Effect of PKA on Ca^{2+} sensitivity in cTn-reconstituted RP. (A) Typical chart recording showing force-pCa protocols. A fiber was treated with cTn, PKA, and sTn, in this order (see Terui et al., 2008 for exchange ratio of Tn subunits under the same condition; i.e., $\sim$50% with cTn reconstitution). Green arrows (blue arrows) indicate percentage of force at pCa 6.0 (pCa 5.75) compared with the maximum obtained at the end of each force-pCa protocol. (B) Force-pCa curves showing the effects of cTn reconstitution, PKA treatment, and sTn reconstitution on Ca^{2+} sensitivity in RP. Black solid line, control with no treatment; gray solid line, cTn reconstitution; red solid line, PKA treatment; black dashed line, sTn reconstitution. (Inset) Summary of pCa_{50} values. *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with cTn reconstitution; †, $P < 0.05$ compared with PKA. $n = 9$. (C) Force-pCa curves showing the effect of PKA on Ca^{2+} sensitivity in RP with no Tn reconstitution. (Inset) pCa_{50} values before and after PKA treatment. Maximal force: 175.35 ± 6.32 and 176.25 ± 5.31 mN/mm^2 ($P > 0.05$) before and after PKA, respectively; n_H: 4.61 ± 0.34 and 4.67 ± 0.30 ($P > 0.05$) before and after PKA, respectively. $n = 7$.

completely replaced cTnI with sTnI, and the ratio of sTnT or sTnI to actin was similar to that observed in control RP before PCRF reconstitution (sTn reconstitution ratio after PCRF reconstitution: sTnI, 107.04 ± 10.54%; sTnT, 85.97 ± 11.76%; residual cTnI, $\sim$0%; $n = 6$).

As shown in the chart recording (Fig. 5 B), submaximal forces (at pCa 6.0 and 5.75) were decreased upon PCRF treatment by a magnitude greater than that of maximal force, resulting in a decrease in the percentage to the maximum. PKA treatment further decreased submaximal forces with little or no change in maximal force. Finally, sTn reconstitution restored submaximal forces to levels similar to those obtained before PCRF reconstitution, accompanied by a slight increase in maximal force.

Force-pCa curves in Fig. 5 C show that Ca^{2+} sensitivity is decreased by 0.13 ± 0.02 pCa units with PCRF reconstitution, and it is further decreased by 0.10 ± 0.02 pCa units with PKA treatment, which is similar to what was observed in cTn reconstitution in RP (see Fig. 4 B). Also, sTn

reconstitution almost completely restored Ca^{2+} sensitivity to the original level before PCRF reconstitution. As with cTn reconstitution, maximal force and n_H were decreased by PCRF reconstitution, but not by PKA treatment, and both were restored with sTn reconstitution to levels not significantly different than those obtained before PCRF reconstitution (Table III).

DISCUSSION

The goal of this study was to provide direct evidence that PKA-based phosphorylation of cTnI has the potential to decrease myofibrillar Ca^{2+} sensitivity associated with cTnC in different types of the striated muscle sarcomere. To test this, we investigated whether PKA induces Ca^{2+} desensitization in PLV and RP muscles, by using thin filament reconstitution. We demonstrated that PKA-dependent cTnI phosphorylation decreased Ca^{2+} sensitivity not only in PLV, but also in RP after reconstitution
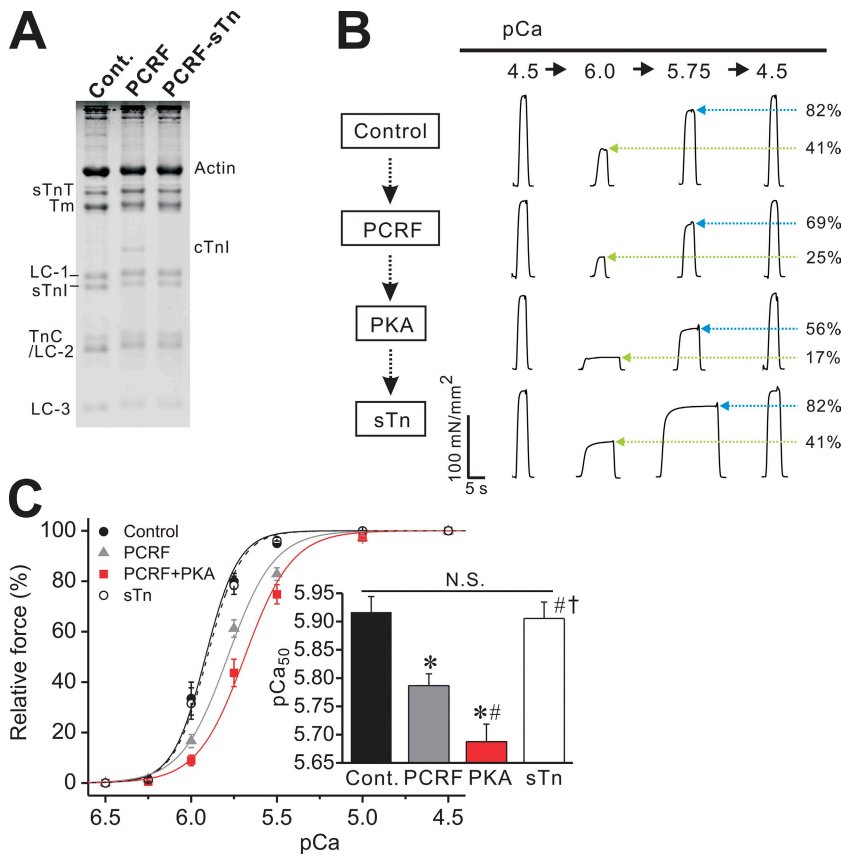
Figure 5. Effect of PKA on Ca^{2+} sensitivity in PCRF-reconstituted RP. (A; Left) 15% gel showing reconstitution of RP with PCRF. Cont., control fibers; PCRF, PCRF-reconstituted fibers; PCRF-sTn, PCRF-reconstituted fibers treated with sTn; LC-3, myosin light chain 3. For other abbreviations, see legend to Fig. 2 A. (B) Typical chart recording showing force-pCa protocols. A fiber was treated with PCRF, PKA, and sTn, in this order. Green arrows (blue arrows) indicate percentage of force at pCa 6.0 (pCa 5.75) compared with the maximum obtained at the end of each force-pCa protocol. (C) Force-pCa curves showing the effects of PCRF reconstitution, PKA treatment, and sTn reconstitution in RP. Black solid line, control with no treatment; gray solid line, PCRF reconstitution; red solid line, PKA treatment; black dashed line, sTn reconstitution. (Inset) Summary of pCa_{50} values. *, P < 0.05 compared with control; #, P < 0.05 compared with PCRF; †, P < 0.05 compared with PKA. $n = 10$.

with the cTn complex. Moreover, the Ca^{2+}-desensitizing effect was independent of the TnT isoform in both types of muscle. The present findings are summarized in Table IV.

In both cardiac and skeletal sarcomeres, TnT is the largest component of the Tn complex. Within the cTn complex, the N-terminal half of cTnT anchors the complex to Tm, and the C-terminal half forms a complex with cTnC, cTnI, and Tm (e.g., Solaro and Rarick, 1998; Ohtsuki and Morimoto, 2008). Despite extensive regions of homology among the various forms of cTnT and sTnT, there are important regions of structural diversity, especially in the N-terminal region, which is highly charged and essentially absent in sTnT (Solaro and Rarick, 1998). In the present study, however, we found that the force-pCa curve was shifted leftward (rightward) by a similar magnitude upon cTn and PCRF reconstitution in PLV (in RP) (Fig. 2, C and D, PLV, and Figs. 4 B and 5 C, RP). Therefore, the TnT isoform variance of adult ventricular versus adult fast skeletal isoform is unlikely to account for the altering of Ca^{2+} sensitivity, as demonstrated earlier in rat (Chandra et al., 2006). Rather, it is likely that the "on–off" equilibrium of the thin filament state is more strongly governed by the TnC–TnI interaction within the Tn molecule.

cTnI is phosphorylated not only by PKA, but also by other kinases, such as various types of PKC, cGMP-dependent protein kinase (PKG), and p21-activated kinases (Pak) (e.g., Layland et al., 2005). Despite conflicting reports regarding the physiological effects of PKC-based phosphorylation of cTnI at Ser43/45 (and Thr144), an emerging consensus based on recent findings is that Ser43/45 phosphorylation reduces maximal Ca^{2+}-activated force, and in concert with Thr144 phosphorylation, it reduces maximal sliding velocity in in vitro motility assays (Layland et al., 2005). PKG phosphorylates cTnI at Ser23/24 at a ~100-fold slower rate than PKA, with a physiological effect similar to that by PKA (Blumenthal et al., 1978; Yuasa et al., 1999; Layland et al., 2005). Pak reportedly phosphorylates cTnI at Ser149, resulting in an increase (not a decrease) in Ca^{2+} sensitivity with no change in maximal force (Buscemi et al., 2002). Also, cTnT, but not sTnT, is phosphorylated by PKC in the C-terminal half of this molecule (Solaro and Rarick, 1998). However, PKC-based cTnT phosphorylation does not reportedly influence Ca^{2+} sensitivity (Noland et al., 1995). Collectively, it is likely that PKA-dependent phosphorylation of cTnI at Ser23/24 is predominant in Ca^{2+} desensitization among the various phosphorylation sites in cTnI and cTnT.

Reconstitution with cTn (Terui et al., 2008) or PCRF in PLV slightly but significantly increased Ca^{2+} sensitivity (Fig. 2, C and D). Because cTnI Ser23/24 phosphorylation was less in cTn- or PCRF-reconstituted PLV than in control PLV (Fig. 2 B), the Ca^{2+} sensitization may result at least in part from lowering of the background

TABLE IV

Summary of this work describing whether PKA exerts its Ca^{2+}-desensitizing effect in PLV and RP with various combinations of Tn subunits

Muscle type	PLV				RP		
	Original	Reconstituted (cTn)	Reconstituted (PCRF)	Reconstituted (sTn)	Original	Reconstituted (cTn)	Reconstituted (PCRF)
TnI-TnC complex	C	C	C	S	S	C	C
TnT	C	C	S	S	S	C	S
Ca^{2+} desensitization (+PKA)	+	+	+	−	−	+	+

"C" and "S" indicate that the Tn subunit (or the complex) is of cardiac or fast skeletal origin, respectively. +, Ca^{2+} desensitization present; −, Ca^{2+} desensitization absent (see Results).

phosphorylation of these residues. Consistent with this view, PP1 treatment reduced the cTnI phosphorylation level (Fig. 3 A) and increased Ca^{2+} sensitivity (Fig. 3 B). Therefore, the Ca^{2+} sensitization after cTn or PCRF reconstitution and PP1 treatment in PLV may result predominantly from dephosphorylation of Ser23/24 in cTnI. This is supported by the finding that PKA increased cTnI phosphorylation (Figs. 2 B and 3 A) and caused a similar magnitude of Ca^{2+} desensitization after cTn or PCRF reconstitution and PP1 treatment (Fig. 3 C), with a position of the force-pCa curve similar to that of control PLV after PKA treatment (Fig. 1 B).

This study revealed that RP became respondent to PKA treatment showing Ca^{2+} desensitization after thin filaments had been reconstituted with cTn (Fig. 4, A and B) or PCRF (Fig. 5, B and C). Because in a control experiment PKA failed to alter Ca^{2+} sensitivity in RP without thin filament reconstitution (Fig. 4 C), the Ca^{2+}-desensitizing effect after cTn or PCRF reconstitution likely results from Ser23/24 phosphorylation in cTnI and subsequent reduction in the affinity of cTnI for cTnC, as well as in the affinity of cTnC for Ca^{2+}. This interpretation is supported by the result that subsequent treatment with sTn after PKA treatment (~100% reconstitution for PCRF [Fig. 5 A] as well as for cTn [Terui et al., 2008]) almost completely restored Ca^{2+} sensitivity to the original level. It is also worthwhile noting that the magnitude of the PKA-induced shift of pCa_{50} was similar with cTn and PCRF reconstitution not only in PLV (Fig. 2, C and D), but also in RP (Figs. 4 B and 5 C). Howarth et al. (2007) proposed that there are "extended" and "bent" conformations in Tn (see Solaro et al., 2008); phosphorylation or dephosphorylation at Ser23/24 in cTnI modulates the equilibrium between the two conformations ("bent" with phosphorylation), similar to that proposed for regulation of smooth muscle myosin by phosphorylation at the N terminus of the regulatory light chain. It is therefore suggested that the phosphorylation-induced "bending" and the ensuing reduction in Ca^{2+} sensitivity occur regardless of the TnT isoform; viz., regardless of the presence or absence of the N-terminal extension of TnT.

cTn or PCRF reconstitution significantly decreased maximal Ca^{2+}-activated force and n_H in RP (Table III),

but the effects were not significant in PLV (Table II). This suggests that the cTnI–cTnC complex shifts the equilibrium of the thin filament state toward the "off" state to a relatively greater degree than its fast skeletal counterpart (Terui et al., 2008; Fukuda et al., 2009). In fact, upon sTn reconstitution, both maximal force and n_H were restored to levels not significantly different than those obtained before reconstitution in RP (Table III). PKA treatment, however, decreased Ca^{2+} sensitivity with no change in maximal force or n_H in both RP (Table III) and PLV (Tables I and II), suggesting a different mode of action between Tn reconstitution and PKA treatment in the thin filament regulation. We consider that Tn reconstitution can potentially alter the near-neighbor regulatory unit interactions within thin filaments (see Terui et al., 2008), whereas PKA-based Ser23/24 phosphorylation in cTnI simply reduces Ca^{2+} binding to cTnC with no change in signaling between neighboring regulatory units. Clearly, future studies are needed to clarify the differences in the effects between isoform switching of Tn subunits and phosphorylation by various kinases.

The magnitude of Ca^{2+} desensitization by PKA in cTn- or PCRF-reconstituted RP was ~0.1 pCa units (Figs. 4 B and 5 C), ~50% of the effect observed in reconstituted PLV (Fig. 2, C and D). This is probably due to the fact that in RP the thin filament reconstitution ratio with either cTn or PCRF cannot technically exceed ~50% to avoid irreversible damages to the regulatory system (see Terui et al., 2008), whereas in PLV thin filament reconstitution can be achieved by ~100% with cTn or PCRF (see Terui et al., 2008 and Fig. 2 A). Given the finding that the magnitude of PKA-based Ca^{2+} desensitization is linearly correlated to the amount of cTnI (Fig. 1 D), it can be considered that the magnitude of Ca^{2+} desensitization is similar in both PLV and RP; i.e., by potentially ~0.2 pCa units with a full amount of the cTnI–cTnC complex.

One may point out that MyBP-C phosphorylation has to be taken into account in the interpretation of the data on PLV due to its potential impact on Ca^{2+} sensitivity. MyBP-C, a large protein associated with the thick filament of striated muscle, constrains cross-bridges to the thick filament backbone (see Granzier and Campbell,

2006; Oakley et al., 2007 and references therein). A binding site for the light meromyosin domain of myosin is present near the C terminus of MyBP-C, and a second site that binds the S2 domain of myosin is present near the N terminus of MyBP-C. The cardiac MyBP-C isoform contains PKA phosphorylation sites within the S2 binding site that are absent in skeletal isoforms. A recent study demonstrated that the primary effect of MyBP-C phosphorylation is to accelerate the cross-bridge cycling rate, and that of cTnI phosphorylation is to reduce Ca^{2+} sensitivity (Stelzer et al., 2007). Our findings that PKA exerted no effect on Ca^{2+} sensitivity after sTn reconstitution in PLV (Fig. 1 C), despite clear enhancement of MyBP-C phosphorylation (Fig. S1), appear to be consistent with the conclusion of the work by Stelzer et al. (2007). It is therefore safe to consider that the PKA-induced Ca^{2+} desensitization in PLV is not modulated by MyBP-C phosphorylation, with and without thin filament reconstitution.

We performed all of the mechanical experiments at SL 2.0 μm, where passive force was barely produced in both PLV and RP. In PLV at longer SL, the decrease in titin-based passive force via PKA-dependent phosphorylation of the N2B segment may weaken titin's effect on the actomyosin interaction (e.g., Cazorla et al., 2001; Fukuda et al., 2001a, 2003), resulting presumably in the enhancement of Ca^{2+} desensitization. However, this possible SL dependence is unlikely to occur in RP due to the absence of the N2B segment (e.g., Prado et al., 2005).

In conclusion, this study for the first time directly demonstrated that PKA-dependent cTnI phosphorylation universally causes Ca^{2+} desensitization in cardiac and fast skeletal sarcomeres, in association with cTnC. Future studies are warranted to investigate whether or not the mechanical properties are altered in response to β-adrenergic stimulation in intact skeletal muscle expressed with the cTnI–cTnC complex.

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