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Effect of Troponin I Phosphorylation by Protein Kinase A on Length-Dependence of Tension Activation in Skinned Cardiac Muscle Fibers

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We examined the effect of troponin I (TnI) phosphorylation by cAMP-dependent protein kinase (PKA) on the length-dependent tension activation in skinned rat cardiac trabeculae. Increasing sarcomere length shifted the pCa (−log[Ca^{2+}])–tension relation to the left. Treatment with PKA decreased the Ca^{2+} sensitivity of the myofilament and also decreased the length-dependent shift of the pCa–tension relation. Replacement of endogenous TnI with phosphorylated TnI directly demonstrated that TnI phosphorylation is responsible for the decreased length-dependence. When MgATP concentration was lowered in the absence of Ca^{2+}, tension was elicited through rigorous cross-bridge-induced thin filament activation. Increasing sarcomere length shifted the pMgATP (−log[MgATP])–tension relation to the right, and either TnI phosphorylation or partial extraction of troponin C (TnC) abolished this length-dependent shift. We conclude that TnI phosphorylation by PKA attenuates the length-dependence of tension activation in cardiac muscle by decreasing the cross-bridge-dependent thin filament activation through a reduction of the interaction between TnI and TnC.

Key Words: **myocardium; calcium sensitivity; troponin I; cyclic AMP-dependent protein kinase; phosphorylation; sarcomere length; troponin C.**

The Ca^{2+} responsiveness of the myofilaments, one of the important physiological determinants of cardiac muscle contractility, change as a function of sarcomere length, as an intrinsic regulatory mechanism; stretching the skinned muscle preparation to a longer sarcomere length increases the Ca^{2+} responsiveness of the myofilaments (length effect) (1). The change in the interfilament lattice spacing, rather than the sarcomere length per se, was shown to be a determinant of the Ca^{2+} responsiveness and Ca^{2+} affinity of troponin C (TnC) in cardiac myofilaments (2). Thus, one possible mechanism for the length effect is that a stretch-induced reduction of the lattice spacing increases the number of attached cross-bridges, which increases the Ca^{2+} affinity of TnC and further facilitates active cross-bridge formation (3–5). This cross-bridge-dependent mechanism is also supported by the studies using intact cardiac muscle preparations (6, 7).

On the other hand, it is widely accepted that β-adrenoceptor stimulation, one of the important extrinsic regulatory mechanisms of cardiac muscle contraction, activates cyclic AMP-dependent protein kinase A (PKA) due to an increase in the intracellular cAMP concentration. PKA phosphorylates troponin I (TnI), an inhibitory subunit of troponin, and decreases the Ca^{2+} responsiveness of the cardiac myofilament (8). Thus, it is an interesting and physiologically important issue how the length effect on the Ca^{2+} responsiveness is influenced when β-adrenoceptor is stimulated.

In the present study, we used chemically skinned trabeculae of rats in order to investigate the possible "cross talk" between the two regulatory mechanisms involving the Ca^{2+} responsiveness (the sarcomere length-dependent regulation and the TnI phosphorylation-dependent regulation), and also to gain an insight into the molecular mechanism of the length effect. Contractile force was produced by increasing the Ca^{2+} concentration ([Ca^{2+}]) (Ca^{2+}-activated tension) or by lowering the MgATP concentration ([MgATP]) (rigor cross-bridge-activated tension); the latter was presumed to reflect direct activation of the thin filaments by rigor cross-bridge formation (9). We studied the roles of TnC and TnI in the length effect by using the following two methods: (i) replacement of intrinsic TnI with phosphorylated TnI by using a troponin exchange technique which exposed the preparation to an excess

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amount of troponin T (TnT) (10, 11); (ii) selective extraction of TnC by using a strong chelator for the divalent cation, *trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid (CDTA) (12). Possible mechanisms of the length effects on tension development and the "cross talk" in cardiac muscle are discussed in relation to the interaction between TnC and TnI.

Some parts of the results have been reported in abstract form (13, 14).

MATERIALS AND METHODS

Preparation of trabeculae. Male Wistar rats (body weight 240–280g) were deeply anesthetized using pentobarbital (50 mg/kg i. p.), and the hearts were quickly excised. The isolated hearts were immediately perfused in a retrograde fashion via the aorta with Tyrode's solution containing bupranolol (β-blocker) (10 μM) and acetylcholine (1 μM). This procedure was essential to maintain a low basal phosphorylation level of TnI. This is probably due to both dephosphorylation of TnI by stimulation of muscarinic cholinergic receptors (15) and to the inhibition of the effects of endogenous catecholamines by bupranolol. Trabeculae were then dissected from the right ventricle and chemically skinned with 1% Triton X-100 for 1 h at 10°C.

Protocols for tension measurement. Both ends of the trabecula (diameter 0.11 ± 0.01 mm, length 1–1.5 mm) were tied with silk monofilaments and connected to a fixed hook and to the arm of a tension transducer (BG-10, Kulite, NJ). Sarcomere length (SL) of the preparation was initially adjusted to 1.9 μm by measuring the laser diffraction lines in the relaxing solution continuously stirred in one of 15 wells of a plastic plate (16). The solution surrounding the preparation was quickly changed to a series of activating solutions (and back to the relaxing solution) by moving the plate horizontally. Ca^{2+} concentration in solutions was sequentially increased from pCa ($-\log[Ca^{2+}]$) 7.0 to 4.8 in a cumulative manner, and then the preparation was allowed to relax in the relaxing solution. For the TnI replacement experiment, the pCa-tension relation was measured twice each at SL 1.9 μm and at 2.3 μm. For the measurement of pMgATP-tension relation, only one measurement run was carried out at each muscle length; data obtained in each experiment were accumulated and analyzed. The temperature of all solutions was maintained at 20.0 ± 0.2°C throughout the experiment.

In order to study the effect of PKA on the length-dependence of tension development, the measurement of either pCa-tension relation or pMgATP-tension relation was repeated four times in each preparation; the first run was at SL 1.9 μm and the second run was at SL 2.3 μm before PKA treatment, and this procedure was repeated after PKA treatment. As to PKA treatment, the preparation was bathed in relaxing solution including PKA (with or without the PKA inhibitor) after the second run for 1 h at 25.0 ± 0.2°C (SL was adjusted to 1.9 μm). A similar protocol was employed for the TnI replacement experiment, which allowed a comparison of the length-dependence in the same preparation.

Since slight damage of the preparation significantly influenced active tension particularly after PKA treatment, we used preparations which preserved maximal developed tension after the PKA treatment more than 80% of that before the PKA treatment at each SL.

TnC extraction and TnI replacement. Cardiac troponin subunits were prepared from both ventricles of rats as described by Tsukui and Ebashi (17), using FPLC ion-exchange column chromatography as described previously (10). Purified TnI was dephosphorylated and phosphorylated by PKA catalytic subunit as described previously (18).

TnI exchange was performed by the method described by Morimoto *et al.* (10). Briefly, the preparation was immersed in a solution containing (mM) 50 MES (2-morpholino ethanesulfonic acid)/KOH (pH 6.0), 250 KCl, 4 EDTA, 0.5 DTT (dithiothreitol), and 1.0 mg/ml purified TnT with continuous stirring for 60 min at 25°C. The preparation was then reconstituted with purified TnI (phosphorylated or dephosphorylated TnI), and TnC at 25°C for 40 min in the relaxing solution containing 0.5 mg/ml of each protein.

Endogenous TnC in the preparation was extracted using CDTA treatment using the method by Morimoto and Ohtsuki (12). The preparation was incubated in a solution containing (mM) 40 Tris, 5 CDTA for 20 min at room temperature. The CDTA-treated preparation was then reconstituted with TnC in the relaxing solution containing 0.5 mg/ml purified TnC for 1 h at 20°C.

Tension data and statistical analysis. At each sarcomere length, the measured tension was normalized to the maximal tension obtained in each measurement. The relationship between relative tension and pCa in each preparation was fitted by non-linear least-squares regression to a Hill equation: relative force = $[Ca^{2+}]^{nH}/(K^{nH} + [Ca^{2+}]^{nH})$, where nH is the Hill coefficient and K is the $[Ca^{2+}]$ which gives half maximal tension. Tension at each pCa was normalized to that produced at pCa 5.1 (the maximal tension). To fit the data of pMgATP-tension relation, a similar equation was used: relative force = $[MgATP]^{nH}/(K^{nH} + [MgATP]^{nH})$. Tension was normalized to that produced at pMgATP 6.0 (maximal tension). The pCa or pMgATP value at 50% relative force (= $-\log K$) was then calculated. Based on average values for nH and pCa50 (or pMgATP50) of each data set, a sigmoidal curve was drawn, which is shown in Figs. 1–4. The measured values were expressed as mean ± standard error of the mean (S.E.M.). For statistical analysis in the case of pCa-tension relation, paired Student's *t*-test was employed with the significance level set at $P < 0.05$, but in the case of pMgATP-tension relations, unpaired Student's *t*-test was used.

Solutions. The concentrations of all solutions used for skinned preparations were calculated using the equilibrium constants reported by Martell and Smith (19), for pH 7.10 at 20°C. Ionic strength was adjusted to 0.2 M using KMS (methanesulfonic acid potassium salt). Solutions with various $[Ca^{2+}]$ contained (mM) 0–10 $CaMS_2$ (methanesulfonic acid calcium salt), 10 EGTA, 20 PIPES (piperazine-N,N′-bis (2-ethanesulfonic acid)), 3.5 MgATP, 1.0 free Mg^{2+}, 10 creatine phosphate, and 10 units/ml creatine phosphokinase. In the calculation of free Ca^{2+}, the apparent dissociation constant of EGTA for Ca^{2+} was set at $10^{-6.4}$ M (16). Solutions for various MgATP concentrations contained (mM) 10 EGTA, 20 PIPES, 0–4.8 MgATP, 1.0 free Mg^{2+}, 10 creatine phosphate, and 150 units/ml creatine phosphokinase; high concentration of creatine phosphokinase was used to maintain the ATP regeneration system and to minimize the accumulation of ADP.

The solution used for PKA-dependent phosphorylation (PKA solution) was similar to that reported by de Tombe (20); (mM) 0.5 EGTA, 20 PIPES, 10 $MgMS_2$, 10 creatine phosphate, 8.2 MgATP, 6 DTT, and 1 unit/ml PKA catalytic subunit, pH 6.8 adjusted with KOH (ionic strength 0.2 M). To inhibit the PKA activity, 2 units/ml protein kinase inhibitor (type III, from porcine heart, Sigma) was added to the PKA solution.

The solution for either tension activation or PKA treatment was used within a month.

RESULTS

Effects of Sarcomere Length and TnI Phosphorylation on pCa-Tension Relation

Figure 1 shows the effects of PKA treatment on the pCa-tension relation. The PKA treatment shifted the pCa-tension relation to the right (desensitization of the myofilaments) at both short (1.9 μm) and long (2.3 μm)

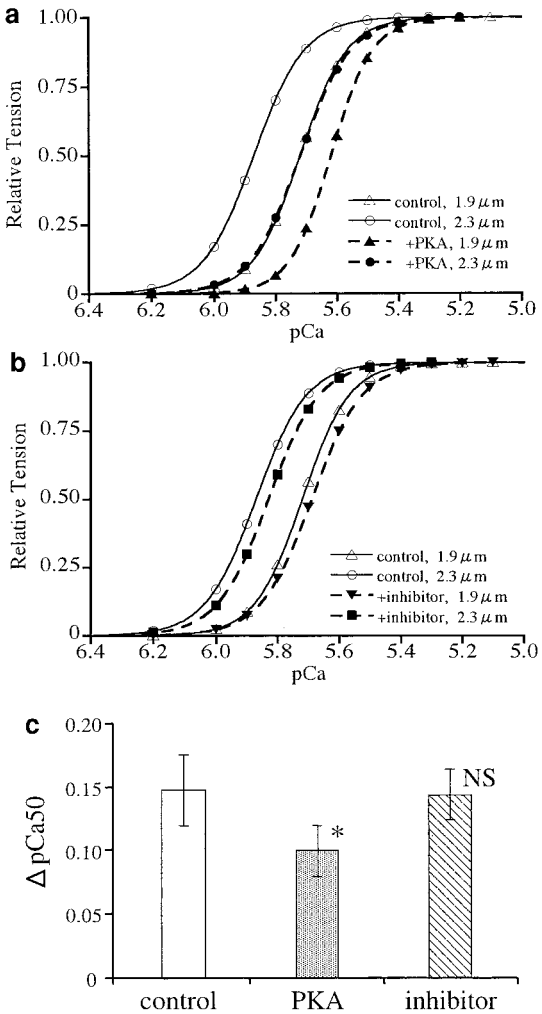

FIG. 1. Effects of PKA treatment in the absence (a) or in the presence (b) of PKA inhibitor on pCa-tension relation. The skinned preparations were incubated in the relaxing solution containing PKA catalytic subunit (1 unit/ml) with or without PKA inhibitor (2 unit/ml) for 1 h at 25°C. In (a) and (b), symbols represent means of data obtained from 4–7 preparations at SL 2.3 μm (○, ●, ■) and at 1.9 μm (△, ▲, ▼). Tension is normalized to that produced at pCa 5.1 (maximal tension). Based on average values for nH and pCa50 of each data set shown in Table 1, sigmoidal curves are drawn. (c) Summary of the length-dependent shift of pCa50 ($_{\Delta p}$Ca50). Each bar represents mean ± S.E.M. Left, control preparations (n = 7); middle, PKA-treated preparations in the absence of inhibitor (n = 7); right, in the presence of PKA inhibitor (n = 4). *$P < 0.05$ compared with control (t-test); NS, not significant.

SLs, but the shift was significantly larger at SL 2.3 μm (Fig. 1a). As a result, the length-dependent shift of the relation was diminished by the PKA treatment; the change in the pCa50 by lengthening ($_{\Delta p}$Ca50) was significantly reduced by the PKA treatment (Fig. 1c and Table 1). These effects of PKA were strongly inhibited by the addition of a PKA inhibitor (Figs. 1b and 1c, see also Table 1). On the other hand, PKA treatment had no significant effects on the maximal Ca^{2+}-activated tension at both SLs; (control vs. PKA-treated preparations), 97.3 ± 8.0 and 92.1 ± 4.7 mg at 1.9 μm (n = 7), and 149.5 ± 16 and 144.9 ± 2.5 mg at 2.3 μm (n = 7).

PKA is known to phosphorylate myosin binding protein C (MyBP-C) as well as TnI. Therefore, in order to eliminate the possible involvement of MyBP-C phosphorylation in the PKA-dependent decrease in the length effect demonstrated in Fig. 1, TnI replacement method was employed in Fig. 2. In this experiment, endogenous TnI was replaced by dephosphorylated or phosphorylated TnI. After being maximally activated at pCa 4.5 and relaxed in the relaxing solution, the preparation was brought into rigor state in the ATP-free solution under acidic and high ionic strength conditions (see Materials and Methods). Addition of an excess amount of TnT released intrinsic TnI and TnC from the preparation, shown as labelled "−(TnI, TnC)," which was due to the replacement of endogenous TnT-TnI-TnC complex with exogenous TnT. Extraction and reconstitution of TnI and TnC was proved by the following observation: (i) nearly maximal tension was produced even in the relaxing solution; (ii) TnC extraction also resulted in the loss of Ca^{2+}-dependence of tension activation, which was shown as a slight Ca^{2+}-activated tension at pCa 4.5; (iii) reconstitution of TnI (by phosphorylated or dephosphorylated TnI, "+TnI") completely suppressed tension development even at pCa 4.5; (iv) Further reconstitution of TnC ("+TnC") restored more than 90% of the Ca^{2+}-activated tension at pCa 4.5; (dephosphorylated TnI vs. phosphorylated TnI), 88.6 ± 8.9 and 85.6 ± 5.2 mg at 1.9 μm (n = 5), and 138.3 ± 18.2 and 131.4 ± 4.3 mg at 2.3 μm (n = 5). Successful reconstitution of troponin subunits by the present protocol has previously been demonstrated by an SDS–PAGE analysis (12). Immunoblot analysis of the dephosphorylated or phosphorylated TnI with antibodies against phosphoserine has also previously been carried out (18), confirming the phosphorylation and dephosphorylation of the TnI used for reconstitution. Also confirmed that the striation uniformity or overall structural integrity of the preparations was well preserved throughout the experiment. The pCa-tension curve measured after TnI replacement clearly showed that the phosphorylation of TnI caused a rightward shift of the relation and also diminished the length-dependent shift of the relation (Fig. 2b, see also Table 1). Qualitatively similar length effects in the PKA treatment and in the direct exchange of phosphorylated TnI into the preparation strongly suggest that TnI phosphorylation is responsible for the diminution of the length-dependence of the pCa-tension relation.

Effects of Sarcomere Length and TnI Phosphorylation on pMgATP-Tension Relation

In order to study whether the cross-bridge-dependent thin filament activation is related to the

TABLE 1

Summary of the Mean Values (±S.E.M.) of pCa50, pMgATP50.nH (Hill Coefficient), ΔpCa50, and ΔpMgATP50 Obtained from the Data of Figs. 1–4 by Fitting to the Hill Equation

	pCa50			nH		pMgATP50			nH	
Treatment	1.9 μm	23 μm	ΔpCa50	1.9 μm	23 μm	1.9 μm	23 μm	ΔpMgATP50	1.9 μm	23 μm
Control	5.72 ± 0.02	5.87 ± 0.04	0.15	5.66 ± 1.02	5.29 ± 0.64	5.40 ± 0.03	5.28 ± 0.03	0.12	4.66 ± 1.08	5.09 ± 1.51
PKA	5.62 ± 0.02*	5.72 ± 0.01*	0.10*	6.39 ± 0.75	5.29 ± 1.22	5.39 ± 0.06	5.39 ± 0.06*	0.00*	5.83 ± 0.99*	6.53 ± 1.21
PKA + inhibitor	5.69 ± 0.02	5.83 ± 0.01	0.14	5.25 ± 0.58	5.30 ± 0.26					
Dephosphorylated TnI	6.20 ± 0.10*	6.40 ± 0.10*	0.20*	1.57 ± 0.58*	1.65 ± 0.67*					
Phosphorylated TnI	6.11 ± 0.10*	6.14 ± 0.11*	0.03*	2.57 ± 0.34*	2.69 ± 0.31*					
−TnC						5.45 ± 0.10	5.42 ± 0.02*	0.03*	6.03 ± 1.25*	4.15 ± 0.73

Note. PKA, after PKA treatment; inhibitor, in the presence of the PKA inhibitor; dephosphorylated TnI, intrinsic TnI was reconstituted with dephosphorylated TnI; phosphorylated TnI, intrinsic TnI was replaced with phosphorylated TnI; −TnC, intrinsic TnC was partially extracted.

*$P < 0.05$ compared with control (t test).

length-dependent shift of the pCa-tension relation after TnI phosphorylation, we studied the length effects on tension development induced by rigor cross-bridges; pMgATP-tension relation was constructed for this purpose as described under Materials and Methods (Fig. 3). Lengthening of the control preparation caused a rightward shift of the relation, indicating that the sarcomere length directly influenced the activation of the thin filament even in the absence of Ca^{2+}. TnI phosphorylation by PKA shifted the relation to the left at SL 2.3 μm, but did not cause a shift at SL 1.9 μm. As a result, the length effect was diminished after the PKA treatment (Fig. 3, see Table 1). Maximal tension due to the activation of the rigor cross-bridge (42.3 ± 3.7% of the maximal Ca^{2+}-activated tension at SL 2.3 μm) was not significantly altered by the PKA treatment at both SL, as in the case of the tension activated by Ca^{2+} (data not shown).

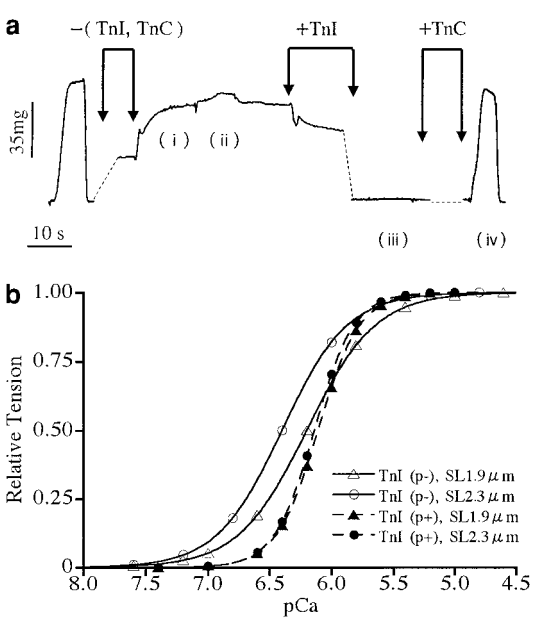

FIG. 2. Effect of TnI reconstitution on length-dependent shift of pCa-tension relation. Skinned preparations were treated with purified TnT (1.0mg/ml) for 1 h at 25°C and reconstituted with phosphorylated or dephosphorylated TnI (0.5mg/ml) and TnC (0.5mg/ml) for 40 min at 25°C. (a) Tension record during the TnI replacement experiment. −(TnI, TnC), TnT treatment in the rigor solution; +TnI, reconstitution with TnI in the relaxing solution; +TnC, reconstitution with TnC in the relaxing solution. (i) The preparation was immersed in the relaxing solution. (ii)–(iv) The preparation was incubated in the activating solution (pCa 4.5). (b), pCa-tension relation at SL 1.9 μm (△, ▲) and SL 2.3 μm (○, ●) after replacement with dephosphorylated TnI (●, ▲, $n = 4$) or phosphorylated TnI (●, ▲, $n = 4$).

Effects of TnC Extraction on pMgATP-Tension Relation

The contribution of TnC to the length effect was then assessed using the preparations in which TnC was selectively extracted (Fig. 4). After the treatment with CDTA, the maximal Ca^{2+}-activated tension (pCa 4.5) was greatly reduced to approximately 10%, suggesting that the major portion of TnC was extracted with this procedure (Fig. 4a). TnC reconstitution restored the maximal Ca^{2+}-activated tension to approximately 70% of the initial level before extraction. When the effects of TnC extraction on the pMgATP-tension relation were examined, an effect similar to the case of PKA treatment was found (Fig. 3); i.e., a larger leftward shift of the relation at the longer SL and diminution of the length effect (Fig. 4b, see Table 1). These results indicate that TnC-TnI interaction plays an important role in the cross-bridge-dependent thin filament activation and that the phosphorylation of TnI by PKA may alter the interaction between TnI and TnC to cause an effect similar to that exerted by TnC extraction on the length dependence.

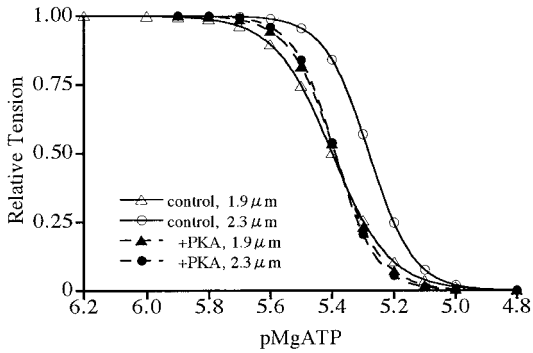

FIG. 3. pMgATP-tension relation at SL 1.9 μm (△, ▲) and SL 2.3 μm (○, ●) in the control (solid lines) and the PKA-treated (dashed lines) preparations**.** The rigor cross-bridge-induced tension was measured with control or PKA-treated skinned preparations by lowering MgATP concentration. Lengthening sarcomere from 1.9 to 2.3 μm significantly shifted the pMgATP-tension relation to the right before PKA application (control) (△, ○, n = 4). The length-dependent shift of the relation was diminished after PKA treatment (+PKA) (▲, ●, n = 4). Tension is normalized to that produced at pMgATP 6.0 (maximal tension). Based on average values for nH and pMgATP50 of each data set shown in Table 1, sigmoidal curves are drawn.

DISCUSSION

The main findings of the present study are the following: (i) the length-dependence of the Ca^{2+}-sensitivity of the contractile elements (measured as a shift in pCa-tension relation) was diminished by TnI phosphorylation, (ii) the rigor cross-bridge-activated tension was also influenced by muscle length, observed as a shift in the pMgATP-tension relation, and this length-dependence was greatly reduced by TnI phosphorylation or by partial extraction of TnC. We used two different methods to prove the effects of TnI phosphorylation in skinned preparations. First, TnI was phosphorylated *in situ* by the addition of PKA. Second, endogenous TnI was substituted with phosphorylated or dephosphorylated TnI by using a technique which replaces endogenous troponin with exogenous troponin with excess TnT treatment. Although PKA phosphorylates myofibrillar proteins other than TnI, such as MyBP-C, the results obtained with the two methods showed a qualitatively similar length-dependent shift in the pCa-tension relation, indicating that the contribution of the phosphorylation of proteins other than TnI to the alteration in the length effect is minor.

Our interpretation regarding the mechanism of TnI phosphorylation effects is the following. A short segment of fast skeletal TnI, which is bound to the actin-tropomyosin (Tm) filament in the absence of Ca^{2+}, has been found to be primarily responsible for the inhibitory effect of TnI and is often called the "inhibitory region" (21). The inhibitory region of TnI is generally considered to act as a molecular switch for muscle contraction; complex formation of the TnI inhibitory region and C-terminal domain of TnC displaces the inhibitory region from the interacting sites of actin-Tm filament, leading to the activation of contraction (22). Thus, the rightward shift of the pCa-tension relation (Fig. 1) and the leftward shift of the pMgATP-tension relation (Fig. 3) caused by TnI phosphorylation might be explained by the enhancement of the inhibitory action of TnI on the thin filament, which is due to an interruption of the interaction between the inhibitory region of TnI and the C-terminal domain of TnC. An increase in sarcomere length decreases the distance between the thick and thin filaments (3–5), which increases the probability of the cross-bridge attachment to the thin filament. Then, attached cross-bridges activate the thin filament and further facilitate the activation of neighboring cross-bridges (23), leading to cooperative development of larger tension. Phosphorylation of TnI or extraction of TnC is considered to enhance the inhibitory effect of TnI on the thin filament as described above; this in turn could antagonize the lengthening-induced thin filament activation, i.e., the enhancement of the cross-bridge formation, resulting in the decrease in the length effect.

In contrast to the present study, previous studies using skinned trabeculae of cardiac muscles showed that TnI phosphorylation by PKA had no effects on the length-dependent shift of the pCa-tension relation (24).

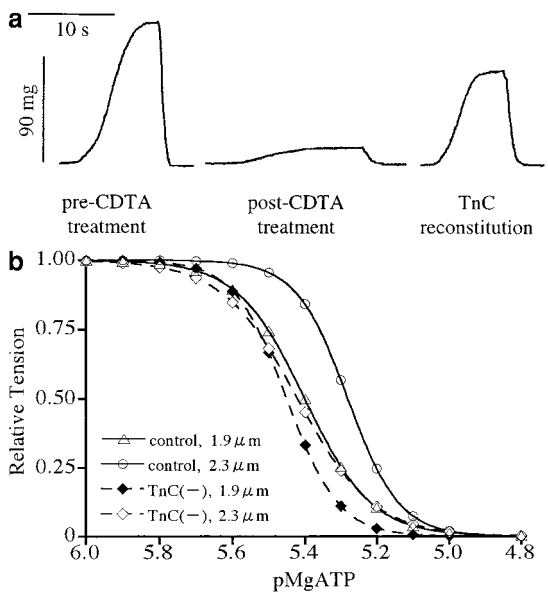

FIG. 4. Effects of TnC extraction on the length-dependent shift of pMgATP-tension relation. For TnC extraction, skinned preparations were incubated in a solution containing 5 mM CDTA for 20 min at 25°C. Then, rigor cross-bridge activated-tension was measured in the absence of Ca^{2+}. (a), chart recordings showing Ca^{2+}-activated tension obtained from a preparation before CDTA treatment (left), after CDTA treatment (middle), and after reconstitution with TnC (right). (b), pMgATP-tension relation at SL 1.9 μm (△, ◆) and SL 2.3 μm (○, ◇) in the control preparations before CDTA treatment (solid lines, n = 4), and the TnC-extracted preparations after CDTA treatment (dashed lines, n = 4).

The reasons for this discrepancy should lie in the differences in the treatment procedure of the heart during dissection. We treated the excised heart with bupranolol (a β-blocker) and acetylcholine (see Materials and Methods). In experiments without this treatment, a much smaller shift was observed in the pCa-tension relation after PKA treatment, suggesting that significant phosphorylation may occur during the time of sacrifice of the animal and dissection probably due to endogenous catecholamines. In addition, it was recently reported that PKA treatment enhanced the length effect in murine skinned single myocytes (25), which was judged by measuring Ca^{2+} concentration (expressed as μM) required for a half maximal tension development. However, if the Ca^{2+} concentrations expressed as μM in the report are converted to pCa unit, the changes in pCa50 at different sarcomere lengths before and after PKA treatment are not significantly different, or rather just the same. Thus, the interpretation of the result is not straightforward and the result is not conclusive.

It was reported that the length-dependent shift of the pCa-tension relation of tetanic contraction measured in aequorin-injected ferret papillary muscles is enhanced by β-adrenoceptor stimulation (26). Two essential experimental conditions, however, are different between the aequorin experiment and the present study; intact or skinned preparation and ferret or rat. We measured the length-dependent shift of pCa-tension relation using skinned ferret trabeculae as in the case of the present experiment. The PKA treatment decreased the length-dependent shift of pCa-tension relation (5 experiments, unpublished data) similar to the present result. Therefore, skinned preparations of rats and ferrets show qualitatively similar results regarding the effect of PKA on the length-dependence of the Ca^{2+} responsiveness. Thus, the difference of preparation (intact or skinned preparation) may be critical for explaining the different results. Furthermore, the procedure for the measurement of pCa-tension relation in intact preparations; intracellular Ca^{2+} concentration and tension in intact preparations were measured at each extracellular Ca^{2+} concentration one by one, but pCa-tension relation in skinned preparations was measured by cumulatively applying Ca^{2+} solutions. Further investigation are required to clarify whether these differences in preparation and experimental procedure are substantial for explaining the results in intact and skinned preparations.

In summary, the present study demonstrated that TnI phosphorylation diminished the length-dependence of Ca^{2+}-activated tension (pCa-tension relation), and both TnI phosphorylation and partial TnC extraction also diminished the rigor cross-bridge-activated tension (pMgATP-tension relation) in skinned cardiac trabeculae. These results indicate that the interaction between TnC and TnI play an important role in the cross-bridge-dependent thin filament activation, and provide first strong evidence that TnI phosphorylation by PKA decreases the length effect on the tension activation in myocardium by reducing this interaction between TnI and TnC.

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