

# Troponin I in the murine myocardium: influence on length-dependent activation and interfilament spacing

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Cyclic AMP-dependent protein kinase (PKA) targets contractile proteins, troponin-I (TnI) and myosin binding protein C (MyBP-C) in the heart and induces a decrease in myofilament  $\text{Ca}^{2+}$  sensitivity. Yet, the effect of sarcomere length (SL) change on  $\text{Ca}^{2+}$  sensitivity (length-dependent activation: LDA) following PKA-dependent phosphorylation is not clear. To clarify the role of PKA-dependent phosphorylation of TnI and MyBP-C on LDA in the heart, we examined LDA in skinned myocytes from a non-transgenic (NTG) and a transgenic murine model in which the native cardiac isoform (cTnI) was completely replaced by the slow skeletal isoform of TnI (ssTnI-TG) lacking the phosphorylation sites for PKA, while retaining PKA sites on MyBP-C. In NTG myocytes, PKA treatment decreased  $\text{Ca}^{2+}$  sensitivity at each SL, but enhanced the impact of SL change on  $\text{Ca}^{2+}$  sensitivity. Despite a greater sensitivity to  $\text{Ca}^{2+}$  and a reduction in LDA, neither  $\text{Ca}^{2+}$  responsiveness nor LDA was affected by PKA treatment in ssTnI-TG myocytes. To determine whether the above observations could be explained by the lateral separation between thick and thin filaments, as suggested by others, we measured interfilament spacing by X-ray diffraction as a function of SL in skinned cardiac trabeculae in the passive state from both NTG and ssTnI-TG models before and following treatment with PKA. Phosphorylation by PKA increased lattice spacing at every SL in NTG trabeculae. However, the relationship between SL and myofilament lattice spacing in ssTnI-TG was markedly shifted downward to an overall decreased myofilament lattice spacing following PKA treatment. We conclude: (1) PKA-dependent phosphorylation enhances length-dependent activation in NTG hearts; (2) replacement of native TnI with ssTnI increases  $\text{Ca}^{2+}$  sensitivity of tension but reduces length-dependent activation; (3) MyBP-C phosphorylation by PKA does not alter calcium responsiveness and induces a decrease in myofilament lattice spacing at all sarcomere lengths and (4) length-dependent activation in the heart cannot be entirely explained by alterations in myofilament lattice spacing.

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A well-studied mechanism by which myocardial function is tuned to the haemodynamic load is through  $\beta$ -adrenoceptor stimulation and subsequent activation of cAMP-dependent protein kinase (PKA). Signalling through this pathway is associated with increased rates of relaxation and contraction in the myocardium (Ross & Sobel, 1972; Luo *et al.* 1994) and is believed to be predominantly due to phosphorylation of phospholamban by PKA leading to enhanced reuptake of  $\text{Ca}^{2+}$  by the sarcoplasmic reticulum (Kranias, 1985; Luo *et al.* 1994; Solaro & Van Eyk, 1996; Wolska *et al.* 1996). PKA additionally targets contractile proteins, specifically troponin I (TnI) and myosin binding protein C (MyBP-C), and results in a decrease in myofilament  $\text{Ca}^{2+}$  sensitivity (Solaro *et al.* 1976; Garvey *et al.* 1988). It has been suggested that phosphorylation of myofilament proteins may affect cardiac contractility independently of the alterations in the dynamics of  $\text{Ca}^{2+}$

handling (Kentish *et al.* 2001). Although a regulatory role for MyBP-C cannot be altogether eliminated in the light of an emerging literature (Kunst *et al.* 2000; Winegrad, 2000), there is good evidence to suggest that the PKA effect in the myocardium is due to phosphorylation of TnI and not MyBP-C (Fentzke *et al.* 1999; Kentish *et al.* 2001).

Other studies have shown that replacement of cardiac TnI (cTnI) with the slow skeletal isoform (ssTnI) in the heart can alter contractile properties of heart muscle by increasing  $\text{Ca}^{2+}$  sensitivity (Fentzke *et al.* 1999) and reducing the response to changes in muscle length (Arteaga *et al.* 2000). The TnI isoforms differ by the presence of a unique 32 amino acid extension at the N-terminus of cTnI. Since this extension contains the two targets for PKA, ssTnI cannot be phosphorylated by PKA (Fentzke *et al.* 1999; Kentish *et al.* 2001). Though the specific role of this N-terminus has not

fully been elucidated, these latter studies implicate TnI as an important modulator of both  $\text{Ca}^{2+}$  sensitivity and length-dependent activation, a phenomenon believed to be the cellular mechanism for the Frank-Starling law of the heart (ter Keurs *et al.* 1980).

The increase in  $\text{Ca}^{2+}$  sensitivity upon an increase in sarcomere length may be due to a decrease in interfilament spacing with an increase in length (Rome, 1968) thereby influencing cross-bridge reactivity (Godt & Maughan, 1981; McDonald & Moss, 1995; Fuchs & Wang, 1996). That is, cross-bridges may be more likely to enter into a strong binding state at a given level of activator  $\text{Ca}^{2+}$  when interfilament spacing is reduced. The correlation between interfilament spacing, alterations in  $\text{Ca}^{2+}$  sensitivity, and length-dependent activation following TnI phosphorylation or TnI isoform composition are not known. Given the significance of sarcomere length, TnI isoform composition and TnI phosphorylation status on myocardial contraction, several questions arise. (1) Does PKA-mediated phosphorylation of cTnI affect the sarcomere length dependency of myofilament activation, and if so, is this effect due to the phosphorylation of cTnI or MyBP-C? (2) What is the role of interfilament spacing in determining myofilament  $\text{Ca}^{2+}$  sensitivity and length-dependent myofilament activation under these conditions?

In experiments reported here, we determined the effect of PKA-mediated phosphorylation on sarcomere length-(SL)-dependent activation in skinned single cardiac myocytes obtained from either non-transgenic mice or transgenic mice in which cTnI was completely replaced by ssTnI in the heart. In addition, interfilament spacing was measured over a wide range of sarcomere lengths in skinned cardiac trabeculae using synchrotron X-ray diffraction. We found that: (1) PKA-mediated phosphorylation induces an increase in SL-dependent activation; (2) this effect is due to phosphorylation of cTnI and not MyBP-C; and (3) alterations in  $\text{Ca}^{2+}$  sensitivity and SL-dependent activation upon PKA-mediated myofilament phosphorylation or TnI isoform composition cannot be predicted, solely, by alterations in interfilament spacing.

## METHODS

### Myocyte preparation

All experiments were performed according to institutional guidelines concerning the care and use of experimental animals. Mice (NTG and ssTnI-TG; 20–30 g) were anaesthetized (sodium pentobarbital 50 mg (kg body weight)<sup>-1</sup> i.p. injection) and the hearts were rapidly excised and retrogradely perfused with a modified Krebs-Henseleit (K-H) solution (de Windt *et al.* 1999) (mmol l<sup>-1</sup>): NaCl 118.5, KCl 5, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 2, ethylenediaminetetraacetic acid, EDTA 0.5, D(+)-glucose 10, NaHCO<sub>3</sub> 25, pyruvic acid 1.5, CaCl<sub>2</sub> 3.0. In addition, insulin (5 u l<sup>-1</sup>) and propranolol (5 μmol l<sup>-1</sup>), to block non-specific β-adrenergic activation, and carbamyl choline (10 μmol l<sup>-1</sup>), to enhance phosphatase activity (Gupta *et al.* 1994) were added to the

perfusate. 3-Butanedione monoxime (20 mmol l<sup>-1</sup>) was also added to the K-H solution to inhibit contraction, presumably through the energetic stabilization of the unattached state of the myosin molecule (Backx *et al.* 1994). Skinned myocytes or myocyte fragments were obtained by mechanical isolation as described previously (Fan *et al.* 1997; Fentzke *et al.* 1999). Briefly, during perfusion with the K-H solution, a section of the left ventricle was minced into 1–2 mm pieces. Next, the pieces were mechanically disrupted at high speed for 15–20 s (Power Gen 700D; Fisher Scientific) in ice-cold standard relaxing solution (mmol l<sup>-1</sup>): Na<sup>+</sup>-ATP 4, MgCl<sub>2</sub> 1, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 2, KCl 100, imidazole 10. The homogenized tissue was then centrifuged at 800 r.p.m. (Marathon 22 Kbr; Fisher Scientific) for 1 min at 4°C. The supernatant was discarded and the tissue was resuspended in standard relaxing solution containing 0.3% Triton X-100 (Pierce Chemical) for 6 min to remove the sarcolemma and all remaining membranous structures. After washing twice with the relaxing solution, the cells and cell fragments were resuspended in standard relaxing solution and stored on ice for < 10 h before data collection.

### Experimental apparatus and protocol

The experiments were performed on the stage of an inverted microscope (Olympus) that allowed visualization of the cell or cell fragments. The cell was attached at either end to a sensitive force transducer (Cambridge model 403A; ~300 Hz resonant frequency) and a high-speed servomotor (Cambridge model 308; ~1 ms 90% step response) with stainless-steel minuten tips and silicone rubber. The sarcomere length was measured by video-microscopy; each horizontal pixel line was transformed by fast Fourier transformation (FFT) into a spatial frequency domain and converted into median sarcomere length across the region (Fan *et al.* 1997). Cell length was adjusted by using the servomotor, which was controlled via computer (Apple PowerPC) using custom-designed software (LabVIEW, National Instruments; Austin, TX, USA). Both minuten tips were connected to X-Y-Z manipulators (Newport) permitting accurate positioning of the attached cell. Cell length and width were measured by video-microscopy. Myocyte thickness was measured by placing a small mirror under a 45 deg angle close to the cell by using a three-dimensional manipulator. The myocyte was suspended above a movable stage that contained several solution wells (temperature controlled at 15°C). Rapid solution changes were made by translating the stage laterally via stage manipulators such that another solution-containing well was brought underneath the cell (Fan *et al.* 1997). The myofilament  $\text{Ca}^{2+}$  sensitivity of tension was characterized at two sarcomere lengths (2.25 and 1.95 μm) by measuring steady-state tension development followed by a rapid slackening to assess total tension. Active tension at each [ $\text{Ca}^{2+}$ ] was the difference between total tension and relaxed, passive tension (passive tension was not measurable at the short sarcomere length and was minimal at the long sarcomere length). Cells that did not maintain 80% of initial maximal tension or a visible striation pattern were discarded. In addition, if the extent of internal shortening due to end-compliance in the preparation was extensive (>100 nm) the data were also discarded.

The ionic strength of the solutions was kept at 180 mmol l<sup>-1</sup> by adding the appropriate amount of potassium propionate. In addition, all solutions contained the following (mmol l<sup>-1</sup>): phosphocreatine 10, N,N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid (BES) 100, leupeptin 0.1, phenylmethylsulphonyl fluoride (PMSF) 0.1, dithiothreitol (DTT) 1 and 4 U ml<sup>-1</sup> creatine

**Table 1. Average values of Hill fit to Ca<sup>2+</sup>-force curves**

Treatment	SL = 1.95 μm			SL = 2.25 μm			ΔEC <sub>50</sub>	ΔpCa <sub>50</sub>
	EC <sub>50</sub> (μM)	pCa <sub>50</sub> (μM)	n <sub>H</sub>	EC <sub>50</sub> (μM)	pCa <sub>50</sub> (μM)	n <sub>H</sub>		
NTG								
-PKA (n = 7)	1.72 ± 0.10	5.77 ± 0.03	3.74 ± 0.35	1.31 ± 0.09†	5.89 ± 0.03	2.91 ± 0.23	0.41 ± 0.04	0.12 ± 0.01
+PKA (n = 7)	3.24 ± 0.14*	5.49 ± 0.02	3.40 ± 0.11	2.45 ± 0.13*†	5.61 ± 0.02	3.8 ± 0.16	0.79 ± 0.07*	0.12 ± 0.01
PKA + i (n = 7)	1.67 ± 0.09	5.78 ± 0.02	3.02 ± 0.26	1.29 ± 0.08	5.89 ± 0.03	2.76 ± 0.25	0.38 ± 0.08	0.11 ± 0.03
ssTnI-TG								
-PKA (n = 8)	1.04 ± 0.05*	5.99 ± 0.02	2.98 ± 0.14	0.78 ± 0.06*†	6.11 ± 0.03	2.72 ± 0.23	0.26 ± 0.03*	0.13 ± 0.02
+PKA (n = 8)	1.10 ± 0.04	5.96 ± 0.02	3.24 ± 0.17	0.84 ± 0.04†	6.08 ± 0.02	2.83 ± 0.14	0.25 ± 0.02*	0.11 ± 0.01

Average Hill fit parameters of the Ca<sup>2+</sup>-force relationships obtained from NTG and ssTnI-TG myocytes before and after treatment with PKA at the sarcomere lengths indicated. (PKA + I, PK inhibitor added to PKA solution). The final columns indicate the average length-dependent changes in EC<sub>50</sub> (ΔEC<sub>50</sub> or ΔpCa<sub>50</sub>) for each muscle type and treatment studied (\*P < 0.01 from values obtained from untreated NTG controls; †P < 0.01 obtained from respective values at the short SL indicating a significant length effect).

phosphokinase. The free Mg<sup>2+</sup> and Mg-ATP concentration was calculated at 1 and 5 mmol l<sup>-1</sup>, respectively. To achieve a range of free [Ca<sup>2+</sup>], activating and relaxing solutions were appropriately mixed with an apparent stability constant of the Ca<sup>2+</sup>-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) complex of 10<sup>6.39</sup> assumed. The pH was adjusted to 7.0 at 15.0 °C with KOH. Force was stored on computer via an A/D converter using the custom software for off-line analysis (10 kHz per channel sampling frequency).

### X-ray diffraction experiments

The overall experimental arrangement, sample preparation and protocol have been described in detail previously (Irving *et al.* 2000). Briefly, experiments were performed on the BioCAT undulator-based beamline at the Advanced Photon Source, Argonne National Laboratory. Following rapid excision, the heart was perfused with the identical K-H solution as described above. Trabeculae were dissected from the right ventricular free wall in both NTG and ssTnI-TG mice and demembrated overnight with 1% Triton X-100 in standard relaxing solution. Preliminary experiments indicated robust PKA-dependent phosphorylation in cardiac trabeculae prepared in this manner (data not shown). For the X-ray studies, trabeculae were mounted between a force transducer and a servo-motor in a small trough, which allowed simultaneous collection of the X-ray patterns and viewing of the striation pattern using a long working distance objective (× 40) of an inverted microscope equipped with a CCD video camera. Low-angle X-ray diffraction patterns were collected on a CCD-based X-ray detector. Spacings between the 1,0 and 1,1 equatorial reflections in the diffraction pattern were converted to d<sub>10</sub> lattice spacings using Bragg's law.

Individual trabeculae were stretched to arbitrary sarcomere lengths between 1.9 and 2.4 μm at the beginning of the experiment. Sarcomere lengths were determined from the video-image as described previously (Irving *et al.* 2000) and checked both before and immediately after the X-ray exposure. Fibre length was then systematically lengthened or shortened to collect at least 10–12 (at least every 50 nm) consecutive data points describing the relationship between sarcomere length and interfilament lattice spacing. Next, an additional 2–4 data points were collected at various sarcomere lengths back along the curve to check for reproducibility or hysteresis. The level of passive tension in the lattice was recorded when the X-ray exposure was taken.

### Data analysis

Each individual Ca<sup>2+</sup>-force relationship was fitted to a modified Hill equation:

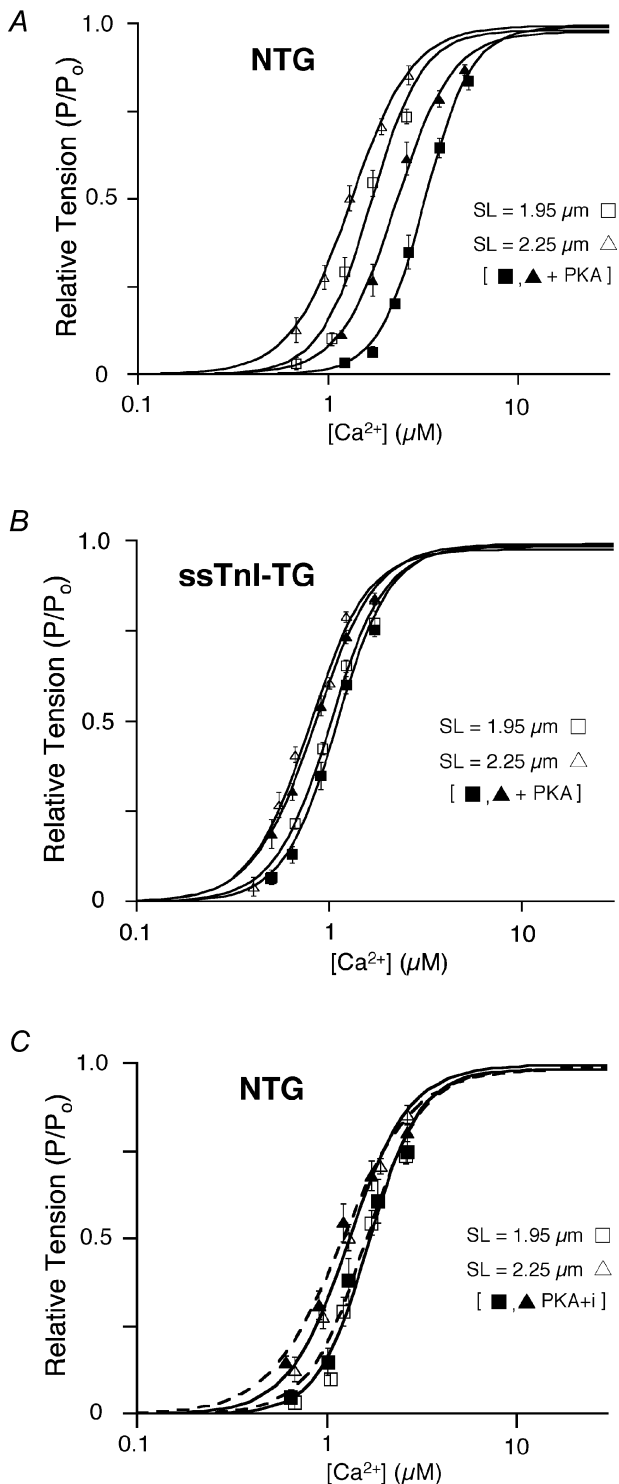
$$F_{rel} = [Ca^{2+}]^{n_H} / (EC_{50}^{n_H} + [Ca^{2+}]^{n_H}),$$

where  $F_{rel}$  is the force relative to maximum Ca<sup>2+</sup> saturated force, EC<sub>50</sub> is the [Ca<sup>2+</sup>] at which force is half-maximal and  $n_H$  is the slope of the Ca<sup>2+</sup>-force relationship (Hill coefficient). The ΔEC<sub>50</sub> was calculated as the difference in EC<sub>50</sub> at SL = 1.95 and 2.25 μm for each experiment. Likewise, the ΔLS was calculated as the difference in LS calculated at SL = 1.95 and 2.25 μm for each experiment. Separation between the symmetrical equatorial 1,0 and 1,1 X-ray diffraction reflections was measured directly from detector images using the program FIT2D (Hammersley, 1998) on a UNIX workstation and converted to d<sub>10</sub> lattice spacing using Bragg's law, which can then, in turn, be converted to the inter-thick filament spacing by multiplying d<sub>10</sub> by 2/√3. The differences among EC<sub>50</sub>, ΔEC<sub>50</sub>, LS and ΔLS for each group at each SL were analysed with a one-way ANOVA Student's *t* test with a *post hoc* Bonferroni correction to assess differences among mean values. All data are shown as means ± S.E.M.

## RESULTS

### Ca<sup>2+</sup> sensitivity of tension

Figure 1 shows the relationship between steady-state force development and free [Ca<sup>2+</sup>] in skinned NTG and ssTnI-TG myocytes before (open symbols) and after (closed symbols) treatment with PKA. The Ca<sup>2+</sup>-force relationships were determined at SL = 1.95 μm (squares) and SL = 2.25 μm (triangles); the continuous lines indicate the best fit of the Ca<sup>2+</sup>-force co-ordinates to a modified Hill equation (see Methods). For both groups, the Ca<sup>2+</sup>-force relationship was shifted to the left at the greater sarcomere length indicating an increase in Ca<sup>2+</sup> sensitivity (a decrease in EC<sub>50</sub>; [Ca<sup>2+</sup>] at which tension is half-maximal) consistent with SL-dependent myofilament activation (Kentish *et al.* 1986). The myofilaments in ssTnI-TG (Fig. 1B) were more sensitive to Ca<sup>2+</sup> than NTG myocytes at both sarcomere lengths, as observed previously (Fentzke *et al.* 1999; Arteaga *et al.* 2000). In addition, the impact of a sarcomere



**Figure 1.  $\text{Ca}^{2+}$ -dependent tension development in cardiac myocytes from NTG and ssTnI-TG hearts**

A and B,  $\text{Ca}^{2+}$ -force relation of NTG (A) and ssTnI-TG (B) cardiac myocytes before (open symbols) and following (closed symbols) treatment with the catalytic subunit of PKA ( $3 \mu\text{g}$  protein (ml standard relaxing solution) $^{-1}$ ) at SL =  $2.25 \mu\text{m}$  ( $\Delta$ ) and SL =  $1.95 \mu\text{m}$  ( $\square$ ). C,  $\text{Ca}^{2+}$ -force relation of NTG cardiac myocytes at two sarcomere lengths before (open symbols) and following (closed symbols) treatment with a PK inhibitor (PKI) added to PKA solution. Lines indicate the Hill fit to the data obtained at the indicated sarcomere length before and following PKA treatment.

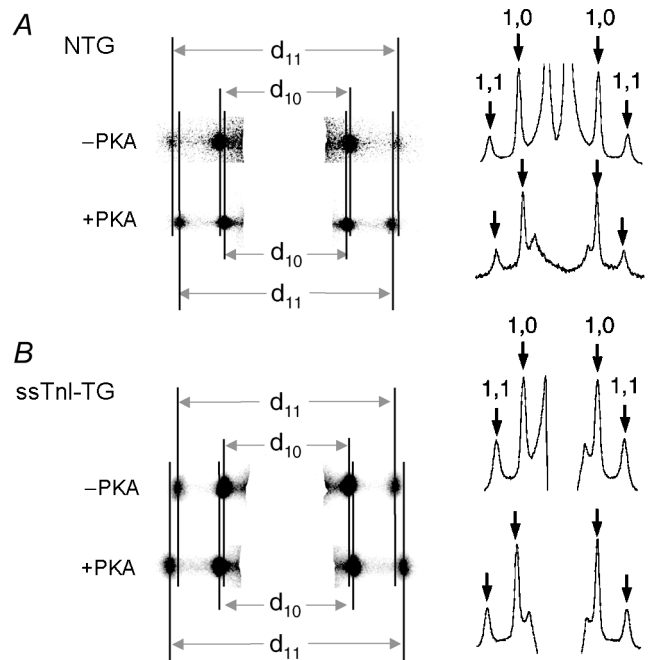
length change on  $\text{Ca}^{2+}$  sensitivity, as calculated as the difference in the  $\text{EC}_{50}$  at each sarcomere length ( $\Delta\text{EC}_{50}$ ), was significantly reduced in ssTnI-TG myocytes. Similar to results reported previously, there was no difference in maximum tension between NTG and TG preparations at either SL (Wolska *et al.* 2001). Earlier studies have employed  $\text{pCa}_{50}$  ( $-\log[\text{Ca}^{2+}]$ ) to index  $\text{Ca}^{2+}$  sensitivity (Godt & Maughan, 1981; McDonald & Moss, 1995; Fuchs & Wang, 1996). Employing this method revealed statistically significant differences in  $\text{Ca}^{2+}$  sensitivity between NTG and ssTnI-TG myocytes. However, length-dependent activation determined as the difference in  $\text{pCa}_{50}$  at the two sarcomere lengths ( $\Delta\text{pCa}_{50}$ ) indicated no significant differences in length-dependent activation between NTG and ssTnI-TG myocytes. The average parameters of the Hill fit from NTG and ssTnI-TG myocytes are summarized in Table 1.

Figure 1 illustrates the effect of treatment of NTG and ssTnI-TG myocytes with the catalytic subunit of PKA ( $3 \mu\text{g}$  protein (ml standard relaxing solution) $^{-1}$ ; Sigma Chemicals). As described in Methods, muscle preparation was designed to prevent additional, non-specific adrenergic phosphorylation or to enhance phosphatase activity resulting in minimal phosphorylation of TnI and MyBP-C. This was supported by robust  $^{32}\text{P}$  incorporation (data not shown) by TnI and MyBP-C in NTG myocardium and MyBP-C in ssTnI-TG preparations, as we have previously shown using this transgenic murine model (Fentzke *et al.* 1999; Kentish *et al.* 2001). Consistent with our previous studies (Solaro, 1995; Janssen & de Tombe, 1997), treatment of the NTG skinned myocardium with PKA induced a rightward shift of the  $\text{Ca}^{2+}$ -force relationship at both sarcomere lengths, as indicated by an increase in the  $\text{EC}_{50}$  (Fig. 1A). In addition, the impact of a sarcomere length change ( $\Delta\text{EC}_{50}$ ) on the  $\text{Ca}^{2+}$ -force relationship was significantly enhanced following PKA treatment, indicating an increase in length-dependent activation properties following PKA treatment. Again, using the  $\text{pCa}_{50}$  parameter, there was a significant decrease in  $\text{Ca}^{2+}$  sensitivity following PKA treatment, yet the effect of length change on  $\text{pCa}_{50}$  ( $\Delta\text{pCa}_{50}$ ) was the same before and following PKA treatment (Table 1). If a protein kinase inhibitor was added to the solution ( $63 \mu\text{g}$  protein (ml standard relaxing solution) $^{-1}$ ; Sigma Chemicals), the effect of PKA was completely abolished independent of parameter choice (Fig. 1C). Furthermore, PKA did not alter maximum tension at either SL, as reported previously (Janssen & de Tombe, 1997).

PKA targets two serine residues (22, 23) at the N-terminus of cTnI, amino acid sites that are not found in ssTnI, and therefore, in ssTnI-TG hearts TnI is not phosphorylated by PKA (Fentzke *et al.* 1999; Kentish *et al.* 2001). However, PKA targets an additional thick filament protein, myosin-binding protein C (MyBP-C; Winegrad, 2000). Treatment of ssTnI-TG myocytes with PKA did not affect  $\text{Ca}^{2+}$  sensitivity of tension at either sarcomere length (Fig. 1B). Consequently,

**Figure 2. Typical X-ray diffraction patterns obtained in skinned cardiac trabeculae from NTG and ssTnI-TG hearts before and following PKA treatment at SL = 2.10  $\mu\text{m}$**

A, CCD images of the diffraction patterns. X-ray diffraction by the myofilament lattice gives rise to two sets of symmetrical, equatorial reflections, delineated by the continuous vertical lines. B, intensity profiles of the 2-dimensional CCD images shown in A. Arrows indicate the peak intensities, corresponding to the 1,0 and 1,1 reflections. These clearly resolved reflections or intensity peaks allowed lattice spacing to be determined within 0.25% accuracy, equivalent to a myofilament lattice spacing resolution of 0.1 nm.

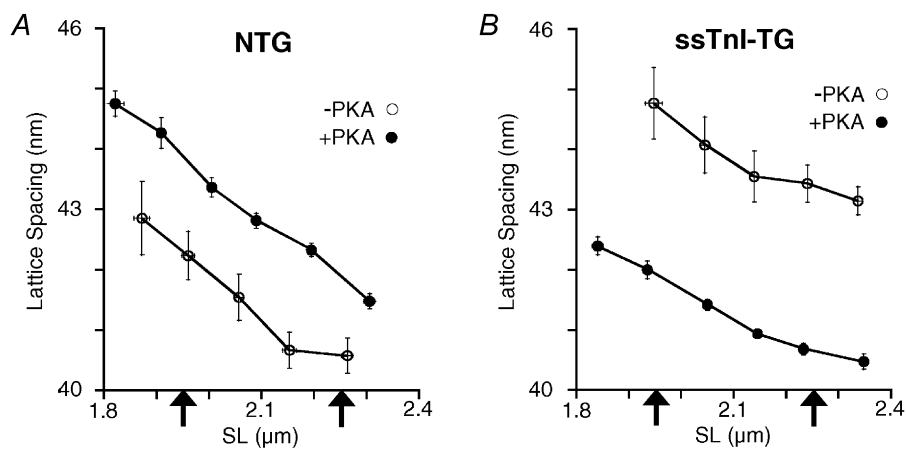


there was no impact on  $\Delta EC_{50}$ , or  $\Delta pCa_{50}$ , and thus no effect on length-dependent activation. These data show that the PKA effect on  $Ca^{2+}$  sensitivity and length-dependent activation in NTG hearts can be attributed, solely, to phosphorylation of TnI and not of MyBP-C. Again, the average parameters of the Hill fit from NTG and ssTnI-TG myocytes, both before and after PKA treatment, are summarized in Table 1.

**Relationship between interfilament spacing and sarcomere length**

To determine the influence of PKA-dependent phosphorylation of cTnI and MyBP-C in the heart on inter-

filament spacing, we simultaneously measured myofilament lattice spacing and sarcomere length in relaxed skinned cardiac trabeculae of NTG and ssTnI-TG mice before and after PKA treatment using synchrotron X-ray diffraction (Irving *et al.* 2000). Figure 2 illustrates typical X-ray diffraction patterns obtained in trabeculae at SL = 2.1  $\mu\text{m}$  from NTG and ssTnI-TG hearts before and after treatment with PKA. These data show clearly resolved symmetrical pairs of 1,0 and 1,1 equatorial X-ray reflections. The adjacent panels illustrate line scans obtained from the 2-D diffraction patterns. Measurement of the distance between the symmetrical sets of reflections, indicated by the continuous vertical lines, allowed lattice spacing to be determined within



**Figure 3. Average myofilament lattice spacing as a function of sarcomere length in skinned cardiac trabeculae from NTG and ssTnI-TG hearts (n = 8–10 for each group)**

The relationships were obtained by averaging the data into 0.05  $\mu\text{m}$  wide sarcomere length bins. A, relationship between lattice spacing and sarcomere length in NTG trabeculae prior to (○) and following (●) treatment with PKA. B, relationship between lattice spacing and sarcomere length in ssTnITG trabeculae prior to (○) and following (●) treatment with PKA. Each arrow indicates the sarcomere length at which the  $Ca^{2+}$ -force relationships were determined.

0.25 % accuracy, equivalent to a myofilament lattice spacing resolution of 0.1 nm (Irving *et al.* 2000). There was an evident inward displacement of the reflections following PKA treatment in NTG trabeculae, indicative of an increased myofilament lattice spacing. In contrast, in ssTnI-TG trabeculae, PKA treatment increased the distance between reflections, indicative of a reduction in myofilament lattice spacing.

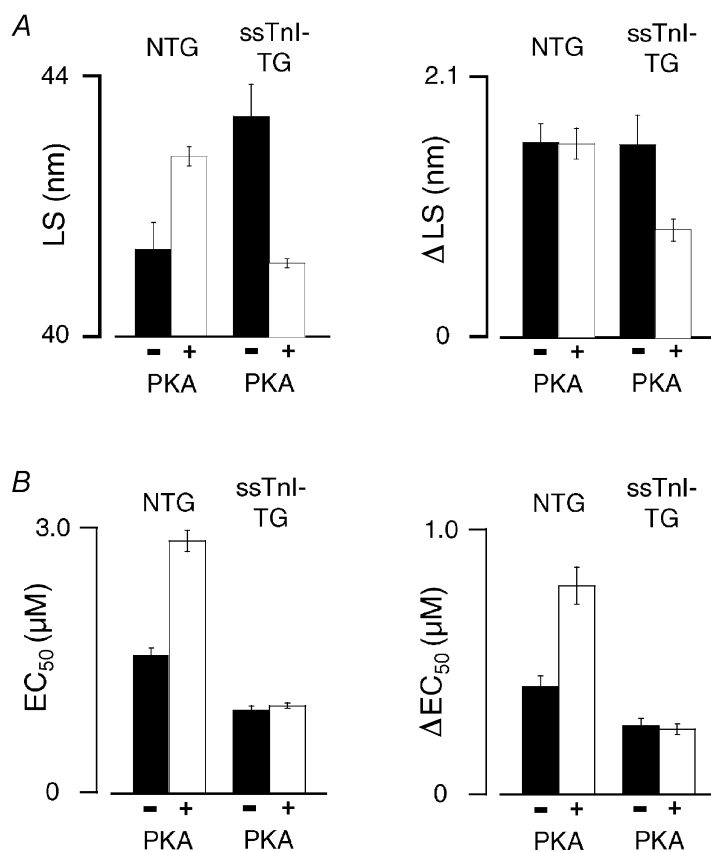
As is shown in Fig. 3, myofilament lattice spacing (LS) was highly dependent on SL, consistent with other and our previous results in mouse myocardium (Fig. 3) (Konhilas *et al.* 2000; Cazorla *et al.* 2001) and in striated muscle (Rome, 1968; Bagni *et al.* 1994; Irving *et al.* 2000). Furthermore, there was a parallel increase in LS at every SL in NTG trabeculae following treatment with PKA (Fig. 3A). Treatment of ssTnI-TG trabeculae, on the other hand, induced contraction of the myofilament lattice at all sarcomere lengths (Fig. 3B). It is also important to note that in both NTG and ssTnI-TG hearts, PKA-dependent phosphorylation greatly reduced the experimental variability among trabeculae, as indicated by a reduction in the standard error of each binned data point. The implication may be that, given the effect of phosphorylation status on LS, there existed a greater variation in basal phosphorylation levels under control conditions (untreated trabeculae). This variation was reduced by PKA treatment, presumably by maximally phosphorylating myofilament proteins. Indeed, preliminary back-phosphorylation

experiments aimed at quantifying this variability in basal phosphorylation status revealed 27 % variation of  $^{32}\text{P}$  incorporation.

To quantify the effects of SL on lattice spacing, lattice spacing was determined at precisely the same sarcomere lengths at which the  $\text{Ca}^{2+}$  sensitivity of tension was measured (SL = 1.95 and 2.25  $\mu\text{m}$ ). The magnitude of the effect of SL change on lattice spacing was defined as the difference in the lattice spacing at these two SLs:  $\Delta\text{LS}$ .  $\Delta\text{LS}$  was similar in untreated NTG ( $\Delta\text{LS} = 1.57 \pm 0.15$  nm) and ssTnI-TG ( $\Delta\text{LS} = 1.56 \pm 0.24$  nm) trabeculae. Furthermore, in NTG hearts, PKA treatment did not alter the effect of SL change on lattice spacing, i.e.  $\Delta\text{LS}$  was similar before and following PKA treatment ( $\Delta\text{LS} = 1.54 \pm 0.12$  nm). However, PKA treatment of ssTnI-TG trabeculae significantly reduced  $\Delta\text{LS}$  ( $\Delta\text{LS} = 0.87 \pm 0.09$  nm) relative to that of untreated ssTnI-TG hearts.

### Relationship between $\text{Ca}^{2+}$ sensitivity and interfilament spacing

In an attempt to correlate  $\text{Ca}^{2+}$  sensitivity and interfilament spacing (left panels, Fig. 4),  $\text{Ca}^{2+}$  sensitivity (bottom panel) and interfilament spacing (top panel) are illustrated in a bar graph at a common sarcomere length (SL = 2.1  $\mu\text{m}$ ; intermediate SL within the working range of the heart) in NTG and ssTnI-TG myocardium before and after PKA treatment. PKA treatment in NTG trabeculae increased LS at SL = 2.1  $\mu\text{m}$  from  $41.3 \pm 0.40$  to  $42.8 \pm 0.15$  nm. On the other hand, in ssTnI-TG trabeculae



**Figure 4. Correlation between length change,  $\text{Ca}^{2+}$  sensitivity and lattice spacing with and without PKA treatment**

A, comparison of lattice spacing (LS) and the effect of length change on lattice spacing ( $\Delta\text{LS}$  in nm). Left, LS at SL = 2.1  $\mu\text{m}$  in NTG and ssTnI-TG trabeculae treated with or without PKA as indicated. Right, the effect of length change on LS ( $\Delta\text{LS}$ ) in NTG and ssTnI-TG trabeculae treated with or without PKA. Error bars are  $\pm$ S.E.M. B, comparison of  $\text{Ca}^{2+}$  sensitivity ( $\text{EC}_{50}$  in  $\mu\text{mol l}^{-1}$ ) and the effect of length change on  $\text{Ca}^{2+}$  sensitivity ( $\Delta\text{EC}_{50}$  in  $\mu\text{mol l}^{-1}$ ). Left,  $\Delta\text{EC}_{50}$  at SL = 2.1  $\mu\text{m}$  in NTG and ssTnI-TG trabeculae treated with or without PKA as indicated. Right, the effect of length change on  $\text{Ca}^{2+}$  sensitivity ( $\Delta\text{EC}_{50}$ ) in NTG and ssTnI-TG trabeculae treated with or without PKA.

PKA treatment induced a reduction in LS from  $43.4 \pm 0.49$  to  $41.1 \pm 0.07$  nm. Whereas PKA treatment significantly reduced  $\text{Ca}^{2+}$  sensitivity in NTG myocytes, PKA treatment did not affect  $\text{Ca}^{2+}$  sensitivity in ssTnI-TG cells. In addition, the right panels of Fig. 4 show the relative impact of a change in SL on  $\text{Ca}^{2+}$  sensitivity (bottom panel) and lattice spacing (top panel), as indexed by the  $\Delta\text{LS}$  and  $\Delta\text{EC}_{50}$  parameters before and after treatment with PKA. Treatment with PKA increased length-dependent activation in NTG trabeculae (increased  $\Delta\text{EC}_{50}$ ), but did not affect the relative impact of SL on lattice spacing ( $\Delta\text{LS}$ ). Treatment of ssTnI-TG skinned trabeculae induced a reduction in  $\Delta\text{LS}$ , but did not affect length-dependent activation ( $\Delta\text{EC}_{50}$ ). If  $\text{Ca}^{2+}$  sensitivity and the length-dependent changes in  $\text{Ca}^{2+}$  sensitivity were directly influenced by changes in interfilament spacing, the relative changes in each  $\text{Ca}^{2+}$  sensitivity parameter should be in parallel with changes in interfilament spacing. From the bar graphs in each group, clearly, changes in interfilament spacing with PKA treatment or with length did not always correlate with changes in myofilament  $\text{Ca}^{2+}$  sensitivity, in either NTG or ssTnI-TG myocardium.

## DISCUSSION

Myofilament activation requires co-operative and allosteric interactions among bound cross-bridges and thin filament proteins (Bremel & Weber, 1972; Swartz & Moss, 1992; Lehrer, 1994; Lehman *et al.* 2000). TnI has been shown to be an important regulatory component during the macromolecular rearrangement of myofilament proteins that results in myofilament force development. Contractile activation is initiated by binding of  $\text{Ca}^{2+}$  to troponin C (Solaro & Rarick, 1998) inducing translocation of tropomyosin (Tm) such that potential cross-bridge binding sites on actin are revealed (Huxley, 1969; Huxley & Simmons, 1971). Strong cross-bridge binding further induces a conformational change in Tm into a third position on actin (Lehman *et al.* 2000; Tobacman & Butters, 2000). In this study, we provide further evidence that TnI plays an integral role in the signalling cascade during myofilament activation. Furthermore, the central mechanisms underlying TnI action on cardiac myofilament activation did not appear to involve interfilament spacing.

### Impact of TnI composition or PKA treatment on $\text{Ca}^{2+}$ sensitivity.

It is well established that phosphorylation of the cardiac sarcomere mediated by protein kinase A (PKA) induces a reduction in myofilament  $\text{Ca}^{2+}$  sensitivity (Solaro *et al.* 1976; de Tombe & Stienen, 1995; Janssen & de Tombe, 1997), an observation that was confirmed in the present study. As for the impact of length change on  $\text{Ca}^{2+}$ -dependent tension development, previous results have been quite variable. A study employing intact electrically stimulated isolated ferret papillary muscle found that treatment with

isoprenaline (an agonist of the  $\beta$ -adrenergic pathway) induced an increase in the muscle length dependency of the intracellular  $[\text{Ca}^{2+}]$ -tension relationship (Komukai & Kurihara, 1997). Others have found either no alteration (van der Velden *et al.* 2000) or a decrease (Kajiwara *et al.* 2000) in length-dependent activation following PKA treatment. In the present study, the impact of sarcomere length on myofilament  $\text{Ca}^{2+}$  sensitivity following treatment with PKA depended on the choice of indexing parameter. Using the  $\text{EC}_{50}$  as the measure of  $\text{Ca}^{2+}$  sensitivity, length-dependent activation, calculated as the difference in  $\text{EC}_{50}$  at the two sarcomere lengths, was enhanced following PKA treatment. However, length-dependent activation determined when  $\text{Ca}^{2+}$  sensitivity was indexed by  $\text{pCa}_{50}$  remained unaffected.

As indicated by our study, the choice of parameter to index myofilament  $\text{Ca}^{2+}$  sensitivity could potentially affect data interpretation. As it stands, the extent of length dependency has a direct impact on the shape of the length-isometric tension relationship in skinned cardiac fibres and the shape of the end-systolic sarcomere length-tension relationship in intact cardiac fibres (Kentish *et al.* 1986). Thus, at a given inotropic state, i.e. a single  $\text{Ca}^{2+}$  concentration, the magnitude of tension development depends on sarcomere length. Similarly, at a given activation level ( $\text{Ca}^{2+}$  concentration), PKA treatment augments isometric tension development following a length change to a greater extent than in muscle fibres in the unphosphorylated state. Since tension is measured at a single concentration, this effect is independent of parameter choice. By reasoning, the use of  $\text{EC}_{50}$  more closely illustrates the physiological impact of PKA-dependent phosphorylation on length-dependent activation. It should be noted that the relation between  $\text{pCa}$  ( $-\log[\text{Ca}^{2+}]$ ) and  $[\text{Ca}^{2+}]$  is non-linear. For these reasons, we believe that the use of the  $\text{EC}_{50}$  parameter to index myofilament  $\text{Ca}^{2+}$  sensitivity is more appropriate than  $\text{pCa}_{50}$ . Nevertheless, whether other factors, besides choice of  $\text{Ca}^{2+}$  sensitivity parameter, play a role in determining the impact of PKA on length dependency of myofilament activation awaits further study.

The length effect notwithstanding, our present study shows that replacement of cardiac TnI (cTnI) with the slow skeletal isoform (ssTnI) in the heart can impart contractile properties of slow skeletal muscle (Konhilas *et al.* 2002a), consistent with previous reports from our laboratory (Fentzke *et al.* 1999; Arteaga *et al.* 2000). The predominant difference between these two isoforms is the presence of a unique 32 amino acid N-terminus extension in cTnI that contains target sites for PKA-dependent phosphorylation (Solaro & Rarick, 1998). Given that PKA targets both cTnI and cardiac MyBP-C and that PKA treatment did not affect either  $\text{Ca}^{2+}$  sensitivity or sarcomere length dependency of  $\text{Ca}^{2+}$  in the ssTnI

transgenic myocardium lacking the PKA phosphorylation sites on TnI, it is likely that the effects of PKA treatment on myofilament activation are due, solely, to phosphorylation of cTnI and not MyBP-C. Phosphorylation of TnI alters the Ca<sup>2+</sup> binding characteristics of TnC (Robertson *et al.* 1982) and the structural interaction between TnI and TnC (Dong *et al.* 1997; Finley *et al.* 1999).

Thus, the changes in Ca<sup>2+</sup> sensitivity and length-dependent activation upon structural modification on the N-terminus of TnI may be due to a change in the kinetics of thin filament protein-protein interactions in the molecular steps leading from Ca<sup>2+</sup> binding to cross-bridge attachment. Although the impact of PKA-mediated phosphorylation in wild-type (non-transgenic) myocardium is well established, the importance of this N-terminus extension in regulation of the cardiac myofilament activation is not entirely clear. It is clear, however, that interfilament spacing plays, at best, only a minor role (see below). Similarly, the physiological function of MyBP-C in muscle contraction is not entirely clear based on these studies. Still, a role for MyBP-C as a regulator of cardiac contractility, in particular Ca<sup>2+</sup> sensitivity, is emerging in the literature (Flavigny *et al.* 1999; Yang *et al.* 1999; Kunst *et al.* 2000; Winegrad, 2000). Finally, the generation of the ssTnI-TG mouse model (Fentzke *et al.* 1999) has provided an important tool to examine the mechanism of length-dependent activation, and to clarify the role of TnI protein isoform and PKA-dependent phosphorylation of cTnI in imparting the phenotypic differences between slow skeletal muscle and cardiac muscle.

### Impact of TnI composition or PKA treatment on interfilament spacing

We also studied the impact of troponin isoform composition and PKA treatment on interfilament spacing by X-ray diffraction techniques. Several observations were apparent from these experiments. First, PKA treatment induced a parallel expansion of the myofilament lattice at all sarcomere lengths in wild-type relaxed skinned trabeculae. That is, phosphorylation by PKA of both cTnI and MyBP-C increased the separation between the thick filaments in equal proportion at each sarcomere length. Second, substitution of cTnI with ssTnI induced a parallel expansion of the myofilament lattice. Third, phosphorylation of MyBP-C, in ssTnI-TG trabeculae, induced a non-parallel contraction of the myofilament lattice such that interfilament spacing became less dependent on sarcomere length following PKA treatment.

Although the factors that determine the separation between thick filaments in the sarcomere are not completely understood, it is clear that electrostatic repulsion of the contractile filaments plays a prominent role in this process (Millman, 1998). It is conceivable that the addition of a negative charge at the N-terminus of cTnI upon PKA treatment induced the expansion of the cardiac myofilament.

Alternatively, or in addition, lattice expansion may be due to the molecular rearrangement of cTnI that has been observed previously upon phosphorylation by PKA (Chandra *et al.* 1997).

The molecular mechanism underlying the expansion in skinned ssTnI-TG trabeculae, however, is less clear. The structural differences between thin filaments containing either ssTnI or cTnI have not been defined. There are charge differences between ssTnI (pI = 9.61) and cTnI (pI = 9.87) making the slow skeletal variant a less positively charged protein than the cardiac variant. Cardiac cTnI has 14 positively charged residues in excess of the negatively charged residues, whereas ssTnI has 11. Moreover, there are charge differences in functionally important micro-domains, which we have proposed are important in the relative insensitivity of TG myofilaments containing ssTnI to deactivation by acidic pH (Li *et al.* 2001; Wolska *et al.* 2001). For example, one potentially significant amino acid difference in cTnI is at Gln157, which exists as Lys124 in ssTnI. These residues are in a region critical to interaction with TnC. Other significant charge differences exist at Ala164, Glu166 and His173 of cTnI, which are replaced by His131, Val133 and Asn140 in ssTnI. Since it is the outermost net negative charge on the thin filament that is most important in regard to lattice expansion (Millman & Nickel, 1980; Millman & Irving, 1988), removing a relatively small amount of positive charge could have a disproportionately large impact by unmasking negative charge at a relatively high radius on the thin filament. Interfilament spacing is much more sensitive to the charge *radius* than to the *amount* of charge (Millman & Nickel, 1980; Millman & Irving, 1988). Hence, without exact knowledge as to the location of the charge, it is difficult to make quantitative predictions regarding the impact on interfilament spacing. Future studies, using specific charge mutants of TnI, could provide insight into the role of specific charge distribution alterations on interfilament spacing.

Perhaps even more difficult to interpret is the non-parallel contraction of the myofilament lattice following PKA treatment of ssTnI-TG trabeculae. Since ssTnI lacks PKA phosphorylation sites, the lattice spacing changes must be attributed to phosphorylation of MyBP-C protein (Garvey *et al.* 1988; Fentzke *et al.* 1999). Despite an overall collapse of the myofilament lattice as observed in this study, it has been shown that phosphorylation of MyBP-C extends the myosin heads away from the backbone of isolated thick filaments and increases their apparent structural order (Weisberg & Winegrad, 1996, 1998). In contrast, preliminary analysis of the d<sub>10</sub>/d<sub>11</sub> reflection intensity ratio from our data suggests a shift of mass away from the thin filament following phosphorylation of MyBP-C. Hence, complex thick filament structural changes may have occurred following phosphorylation of MyBP-C under our conditions.



In any case, phosphorylation of MyBP-C in the presence of ssTnI on the thin filament appears to inhibit compression of the myofilament lattice upon an increase in sarcomere length. Whether this is purely the result of a structural change, a change in charge distribution, or a combination of both phenomena cannot be determined from the current study. Given the effect of phosphorylation on TnI (Chandra *et al.* 1997) coupled with the effect of net charge on lattice spacing, it is reasonable to propose that the structural and/or charge change of MyBP-C following PKA-mediated phosphorylation in the presence of a distinct TnI (ssTnI) could have a non-parallel consequence to the myofilament interfilament spacing–sarcomere length relationship.

Interfilament spacing did not correlate with  $\text{Ca}^{2+}$  sensitivity (Fig. 4). This result suggests that the contribution of interfilament spacing to length-dependent activation of the cardiac sarcomere is negligible, consistent with our recent study in skinned rat cardiac trabeculae (Konhilas *et al.* 2002b). The increase in length-dependent activation following PKA treatment indicates that phosphorylation regulates the signalling between thin filament proteins such that transmission of the length signal is promoted, perhaps by a release of a prevailing limitation that may be imposed upon the co-operative influence of cross-bridge attachment. Likewise, the reverse of this same molecular mechanism may cause the reduction of length signal transmission in face of a lack of the N-terminus extension on troponin I.

### Implications for the ventricular Frank-Starling law relation

Our results have implications with regard to the slope of the relation between end-systolic pressure (ESP) and end-systolic volume (ESV), which is rooted in length-dependent activation and is a manifestation of the Frank-Starling mechanism. We found that length dependence of activation is a variable function of the state of phosphorylation of cTnI. At the short SL, desensitization following PKA treatment was greater than at longer SL. As a result the  $\Delta\text{EC}_{50}$  was greater after PKA phosphorylation than before. We (Arteaga *et al.* 2000) have predicted, on the basis of a simple model relating length-dependent activation to the slope of the ESP–ESV relation, that this ‘disproportional desensitization’ would result in a change in slope. In preliminary experiments (Nowak *et al.* 2001), we have tested this idea using a conductance catheter and comparing the ESP–ESV relation in ssTnI-TG hearts with NTG hearts beating *in situ* in the presence of isoprenaline stimulation. In this case, there was a correlation between the changes in length-dependent activation and the ESP–ESV relation.

### Limitations

In this study we determined active  $\text{Ca}^{2+}$ -dependent force development in single skinned myocytes, while interfilament

spacing was measured in relaxed skinned cardiac trabeculae. It has been illustrated in skeletal muscle that skinned fibres in a rigor state possess an interfilament spacing that is smaller than that of the same muscle fibre under relaxed conditions (Brenner & Yu, 1985; Kawai *et al.* 1993). The suggestion is that the measured lattice spacing in relaxed muscle at a particular SL may not functionally be equivalent to that of activated fibres at the same SL. Thus, although a role for interfilament spacing in the regulation of cardiac muscle contraction appears unlikely, it cannot altogether be excluded based on the present study. Differences in  $\text{Ca}^{2+}$  activation properties between skinned myocytes and skinned cardiac trabeculae are not likely to have affected our data; we found similar values for  $\text{Ca}^{2+}$  sensitivity and length-dependent activation in either skinned papillary or RV trabeculae preparations from either NTG or ssTnI-TG mice (Konhilas *et al.* 1998; Arteaga *et al.* 2000). Also, our study employed skinned muscle preparations in all protocols. It has been suggested that length-dependent activation is less pronounced in intact myocardium as compared with skinned myocardium (Komukai & Kurihara, 1997), despite a similar impact of sarcomere length on interfilament spacing (Irving *et al.* 2000). Hence, there appear to be differences between intact and skinned muscle, such that our results on skinned muscle preparations may not be applicable to intact muscle.

Finally, our data suggest that phosphorylation of either cardiac TnI or MyBP-C may have a large, and possibly opposite, impact on interfilament. Although preliminary studies indicate similar phosphorylation levels of C-protein in NTG and ssTnI-TG animals, we cannot exclude other, compensatory phosphorylation-induced myofilament lattice expansion, as opposed to the lack of the N-terminus extension in ssTnI-TG myocardium. In addition, the small, but variable, level of basal phosphorylation in NTG myocardium may have caused us to overestimate length-dependent activation and  $\text{Ca}^{2+}$  sensitivity in NTG myocardium that would have existed in the absence of basal phosphorylation.

However, it is unlikely that this phenomenon would hamper the comparison between NTG and ssTnI-TG  $\text{Ca}^{2+}$  sensitivity and length-dependent activation. We have recently observed in preliminary experiments (Smith *et al.* 2002) that extraction–reconstitution of either cTnI or ssTnI in skinned rat myocardium resulted in differences in myofilament  $\text{Ca}^{2+}$  sensitivity and length-dependent activation similar to those reported here and in our previous study (Fentzke *et al.* 1999). Finally, recent studies indicate additional myofilament targets (titin and myomesin) for PKA-dependent phosphorylation (Obermann *et al.* 1998; Yamasaki *et al.* 2002). The studies described in this paper have not addressed the role that these myofilament proteins may play and thus cannot be excluded as potential regulators of the PKA effect.

## Conclusions

In the present study, we found that treatment of skinned myocardium with PKA induced a reduction in  $\text{Ca}^{2+}$  sensitivity and an increase in sarcomere length dependency of contractile activation. This effect was due, solely, to phosphorylation of cardiac troponin I (cTnI), not cardiac MyBP-C. In addition, complete substitution of cTnI by ssTnI in the cardiac sarcomere induced an increase in  $\text{Ca}^{2+}$  sensitivity concomitant with a decrease in sarcomere-length dependency of myofilament activation. Both PKA treatment and TnI isoform composition had a profound and opposite impact on interfilament spacing. Interfilament spacing, however, did not appear to mediate alterations in either myofilament  $\text{Ca}^{2+}$  sensitivity or the sarcomere length dependency of myofilament activation.

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