Troponin I chimera analysis of the cardiac myofilament tension response to protein kinase A

MARGARET V. WESTFALL, IMMANUEL I. TURNER, FARIS P. ALBAYYA, AND JOSEPH M. METZGER

Department of Physiology, School of Medicine, University of Michigan, Ann Arbor, Michigan 48109-0622

Received 8 June 2000; accepted in final form 8 September 2000

Westfall, Margaret V., Immanuel I. Turner, Faris P. Albayya, and Joseph M. Metzger. Troponin I chimera analysis of the cardiac myofilament tension response to protein kinase A. Am J Physiol Cell Physiol 280: C324–C332, 2001.—Viral-mediated gene transfer of troponin I (TnI) isoforms and chimeras into adult rat cardiac myocytes was used to investigate the role TnI domains play in the myofilament tension response to protein kinase A (PKA). In myocytes expressing endogenous cardiac TnI (cTnI), PKA phosphorylated TnI and myosin-binding protein C and decreased the Ca2+ sensitivity of myofilament tension. In marked contrast, PKA did not influence Ca2+-activated tension in myocytes expressing the slow skeletal isoform of TnI or a chimera (N-slow/card-C TnI), which lack the unique phosphorylatable amino terminal extension found in cTnI. PKA-mediated phosphorylation of a second TnI chimera, N-card/slow-C TnI, which has the amino terminal region of cTnI, caused a decrease in the Ca2+ sensitivity of tension comparable in magnitude to control myocytes. Based on these results, we propose the amino terminal region shared by cTnI and N- card/slow-C TnI plays a central role in determining the magnitude of the PKA-mediated shift in myofilament Ca2+ sensitivity, independent of the isoform-specific functional domains previously defined within the carboxyl terminal backbone of TnI. Interestingly, exposure of permeabilized myocytes to acidic pH after PKA-mediated phosphorylation of cTnI resulted in an additive decrease in myofilament Ca2+ sensitivity. The isoform-specific, pH-sensitive region within TnI lies in the carboxyl terminus of TnI, and the additive response provides further evidence for the presence of a separate domain that directly transduces the PKA phosphorylation signal.

gene transfer; myocyte; thin filament

β-ADRENERGIC ACTIVATION of protein kinase A (PKA) in cardiac myocytes reduces the Ca2+ sensitivity of myofilament tension (15), and this response is hypothesized to contribute to accelerated relaxation in the intact heart (42). One myofilament protein phosphorylated in response to cAMP-dependent PKA activation is cardiac troponin I (cTnI; Ref. 8), a key regulatory protein of the thin filament. Recent investigations indicate that cTnI phosphorylation plays an important role in PKA-mediated decreases in myofilament Ca2+ sensitivity of tension (3, 11, 15). The increase in cTnI phosphorylation and decrease in myofilament Ca2+ sensitivity of tension typically observed with PKA activation (15) are both markedly attenuated in myocardium expressing the slow skeletal isoform of TnI (ssTnI), which lacks the PKA phosphorylation domain (3, 11). The relationship between cTnI phosphorylation and myocardial function appears to be clinically significant, as there is accumulating evidence that patients experiencing heart failure also have altered levels of phosphorylated cTnI (4, 5). This clinical observation underscores the significance of gaining a better understanding of the role phosphorylated TnI plays in the myofilament response to β-adrenergic stimulation. An important step toward this goal is to investigate the domain(s) within cTnI that are responsible for PKA-induced decreases in the Ca2+ sensitivity of myofilament tension.

At present, cTnI amino acid residues phosphorylated by PKA are well known, but the region(s) within TnI that may be important for the ensuing changes in myofilament function are not well understood. The primary residues phosphorylated by PKA are serine residues 23 (Ser-23) and 24 (Ser-24) within rodent (43) and human cTnI (24). These serines reside within a unique 32-amino acid amino terminal cTnI extension that is absent in slow or fast skeletal TnI isoforms (19). Al-Hillawi and colleagues (1) proposed that phosphorylation of the amino terminal extension of cTnI by PKA leads directly to changes in the interactions between this region of TnI and other myofilament proteins. Spectroscopic analysis of TnI interactions with TnC supports these hypotheses (12). In contrast, results from fluorescence emission studies indicate that PKA phosphorylation of the amino terminal extension of cTnI by PKA leads to changes in the interactions between this region of TnI and other myofilament proteins. Biochemical studies, this region acts as a molecular switch that

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
toggles from actin to troponin C with increasing Ca^{2+} levels (see Ref. 30 for review). Thus phosphorylation of the amino terminal extension of cTnI could affect this isoform-specific, molecular switch domain as a means of influencing myofilament tension. Clearly, functional studies in the intact myofibril are necessary to determine the contribution of these different TnI domains to PKA-induced changes in the myofilament Ca^{2+} sensitivity of tension.

Viral-mediated gene transfer to adult cardiac myocytes was used in the present study to investigate the domain(s) within cTnI responsible for changes in myofilament tension after PKA activation. This approach has previously been shown to result in virtually complete replacement of endogenous cTnI with the ssTnI isoform or individual TnI chimeras without detectable changes in sarcomere architecture, contractile protein stoichiometry, or the isoform expression pattern of other contractile proteins (35, 36, 38). In addition, there is no detectable influence on myofilament function after viral-based gene transfer of the endogenous cTnI isoform (35, 36). Collectively, these findings provide strong evidence for the specificity of myofilament gene transfer. Importantly, gene transfer of TnI isoform/chimeras has provided important new insights into the function of TnI within intact myofibrils of adult myocytes (35, 36, 38). Measurements of myofilament tension in adult myocytes expressing either ssTnI or one of the TnI chimeras constructed from cTnI and ssTnI indicate that there is a hierarchy of myofilament Ca^{2+} sensitivity of tension (35, 36, 38). This hierarchy is best explained by the presence of separate amino and carboxyl terminal TnI isoform-specific domains that influence myofilament Ca^{2+} sensitivity of tension, whereas earlier investigators predicted only a carboxyl terminal region influenced Ca^{2+} sensitivity (10, 32, 34). A previously unknown carboxyl terminal domain responsible for acidic pH-induced decreases in submaximal tension also is observed (see Fig. 1). Acute genetic engineering of TnI is now used here to analyze the domain(s) within TnI mediating the PKA-induced decrease in myofilament Ca^{2+} sensitivity of adult cardiac myocytes.

Experiments in the present study were designed to determine whether the isoform-specific amino terminal region of cTnI coupled to the carboxyl terminus of either TnI isoform is sufficient to account for the direction and magnitude of the PKA-mediated decrease in myofilament Ca^{2+} sensitivity. This possibility can now be differentiated from an alternative possibility that isoform-specific domains within the carboxyl terminal region influence these aspects of the myofilament tension response to PKA in adult myocytes. The relative contribution of an isoform-specific domain in the amino terminus of cTnI was evaluated by expressing a phosphorylatable TnI chimera (N-card/slow-C TnI), which contains the amino terminus of cTnI and carboxyl terminus of ssTnI (see Fig. 1), in adult myocytes. The shift in myofilament Ca^{2+} sensitivity caused by PKA in myocytes expressing this chimera was then compared with the shift observed in myocytes expressing cTnI, ssTnI, or a TnI chimera with the amino terminus of ssTnI, which lacks the amino terminal phosphorylation sites (N-slow/card-C TnI). Additional insight into the region(s) of TnI involved in the myofilament response to PKA was obtained by analyzing the desensitizing effects of PKA phosphorylation in the presence of acidic pH. The additive effect of PKA-mediated TnI phosphorylation and acidosis observed in the present study signals that PKA operates via a region that is separate from the isoform-specific region of TnI affected by acidic pH.

EXPERIMENTAL PROCEDURES

Mutagenesis Strategy

Full-length wild-type ssTnI and cTnI cDNAs were used to generate the TnI chimeras, as described in earlier studies (35, 36). The TnI chimera designated N-slow/card-C TnI (35) is composed of the amino terminus of ssTnI joined to the carboxyl portion of cTnI, and a second chimera (36), designated N-card/slow-C TnI, consists of the amino terminus of cTnI and the carboxyl terminus of ssTnI. The N-slow/card-C TnI chimera is composed of the 68 amino terminal amino acids of ssTnI and carboxyl terminal 110 amino acids of cTnI, whereas the 99 amino acids from the amino terminus of cTnI are joined to the carboxyl terminal 120 amino acids of ssTnI to form N-card/slow-C TnI. The alignment of the four proteins relative to one another is shown in Fig. 1.

Generation of Adenoviral Vectors

Recombinant adenovirus vectors were constructed by cotransfecting shuttle plasmids containing TnI cDNAs (cTnI, ssTnI, or a TnI chimera with the amino terminus of ssTnI, which lacks the amino terminal phosphorylation sites (N-slow/card-C TnI). Additional insight into the region(s) of TnI involved in the myofilament response to PKA was obtained by analyzing the desensitizing effects of PKA phosphorylation in the presence of acidic pH. The additive effect of PKA-mediated TnI phosphorylation and acidosis observed in the present study signals that PKA operates via a region that is separate from the isoform-specific region of TnI affected by acidic pH.

![Fig. 1. Alignment of protein domains for the cardiac troponin I (cTnI) and slow skeletal troponin I (ssTnI) isoforms and the N-slow/card-C TnI and N-card/slow-C TnI chimeras. The primary and secondary Ca^{2+}-sensitive domains previously determined from studies with cTnI-, ssTnI-, N-slow/card-C TnI-, and N-card/slow-C TnI-expressing myocytes are shown (35, 36), along with the protein kinase A (PKA)-mediated phosphorylation domain present in cTnI and N-card/slow-C TnI but not in ssTnI or N-slow/card-C TnI.](http://ajpcell.physiology.org/)
ssTnI, N-slow/card-C TnI, and N-card/slow-C TnI) and pJM17 into HEK 293 cells as described in detail previously (37, 38). Recombinant virus was confirmed by Southern blot analysis (35, 36). High titers of plaque-purified adenoviral stocks were prepared from plaque-purified cellular lysates with a CsCl gradient followed by dialysis in PBS with 10% glycerol for 24 h. Aliquots of virus were stored at −80°C.

Primary Cultures of Rat Ventricular Myocytes

Ventricular myocytes were isolated from adult female rats as described by Westfall et al. (37). An aliquot of Ca2+-tolerant myocytes (2×10⁶ myocytes) was then plated on laminin-coated coverslips and incubated at 37°C in DMEM containing 5% FBS, 50 μl penicillin, and 50 μg/ml streptomycin for 2 h. Cells were then incubated with recombinant adenovirus in DMEM plus penicillin/streptomycin. An aliquot of DMEM plus penicillin/streptomycin (2 ml) was added to each coverslip after 1 h, and serum-free medium was changed the day after adding virus and then every 2–3 days up to 7 days of culture.

Phosphorylation of Myofilament Proteins in Permeabilized Cardiac Myocytes

Myocytes cultured for 6–7 days were permeabilized in 0.1% Triton X-100 for 1 min and rinsed three times in pCa 9.6 relaxing solution (RS; see below). Phosphorylation experiments were initiated by adding 75 μl of RS containing 10 μCi [γ-32P]ATP with or without calyculin A (50 nM) and/or the catalytic subunit of PKA (75 units) to coverslips for 10- to 30-min incubations at room temperature. After coverslips were rinsed in ice-cold RS, myocytes were scraped into sample buffer, and proteins were promptly separated by gel electrophoresis. Phosphorylation experiments in which iso- proterenol was used to stimulate PKA were initiated by adding 2 ml of DMEM plus penicillin/streptomycin containing 50 μCi of [32P]orthophosphate and incubating at 37°C. After 1.5 h, the labeling media was aspirated, and unlabeled DMEM plus penicillin/streptomycin containing 100 nM calyculin A with or without 100 nM isoproterenol was added for 10 min, and then the media was replaced with ice-cold RS. Myocytes were subsequently permeabilized in 0.1% Triton X-100, rinsed three times in RS, and then scraped in sample buffer for separation of proteins by gel electrophoresis.

Analysis of Protein Composition by Gel Electrophoresis and Western Blots

Gel electrophoresis. Cultured ventricular myocytes from coverslips were collected in sample buffer 6–7 days after plating and were separated by gel electrophoresis. Fiber segments of rat soleus and rabbit psoas muscles were collected as described previously (23). Samples labeled with 32P were boiled for 2 min, sonicated for 10 min, and briefly centrifuged. Proteins were then separated by SDS-PAGE and silver stained as previously described in detail (13). Dried gels were scanned (Scannaker 4; Mirotek), and individual radiolabeled protein bands were detected with a PhosphorImager. Scanned gels and the extent of phosphorylation were quantified using Multi-analyst software (Bio-Rad, Hercules, CA).

Western blot analysis. Cultured ventricular myocytes from coverslips were collected in sample buffer 6 days after plating and separated by gel electrophoresis as described above. Proteins were then transblotted onto a polyvinylidene difluoride membrane, and immunodetection was carried out on blots fixed in glutaraldehyde (39). TnI isoform/chimera composition was determined using a 1:4,000 dilution of the primary anti-TnI monoclonal antibody MAB 1691 (Chemicon, Temecula, CA), which recognizes all striated muscle isoforms from rat, as well as the two TnI chimeras (35, 36).

Measurement of Ca2+-Activated Tension in Single Cardiac Myocytes at pH 7.0 and 6.2

Solutions and preparation of samples for mechanical studies. Complete details of the experimental chamber and attachment procedure for mounting single, rod-shaped cardiac myocytes has been reported elsewhere (23). The relaxing and activating solutions used for experiments contained 7 mM EGTA, 20 mM imidazole, 1 mM free Mg2⁺, 14.5 mM creatine phosphate, and 4 mM MgATP with sufficient KCl to yield a total ionic strength of 180 mM. Solution pH was adjusted to 7.00 or 6.20 with KOH/HCl. The RS had a pCa (−log[Ca²⁺]) of 9.0, whereas the pCa of the solution for maximal activation was 4.0. The computer program of Fabiato (9) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, employing the stability constants listed by Godt and Lindley (14).

Cultured cardiac myocytes were permeabilized by brief treatment with 0.1% Triton X-100, washed repeatedly in RS, and then attached to a force transducer (model 403A; Cambridge Technology, Watertown, MA) and a high-performance moving coil galvanometer (model 6350; Cambridge Technology) via glass micropipettes (23). Sarcomere length was set at 2.1 μm. The experimental temperature was set at 15°C because preparation viability decreases and sarcomere length nonuniformity increases more rapidly at higher temperatures.

Measurement of steady-state isometric tension-pCa relationship. Ca2+-activated tension was measured in single myocytes by allowing steady-state isometric tension to develop at each pCa, followed by rapid slackening to obtain the tension baseline. Myocytes were subsequently returned to the original cell length and placed in RS. The difference between developed tension and the tension baseline after the slack step was measured as total tension. Active tension was obtained by subtracting resting tension measured at pCa 9.0 from total tension. Tension-pCa relationships were constructed by expressing tension at various submaximal Ca²⁺ concentrations as a fraction of tension at maximal activation (pCa 4.0). Every third or fourth activation was carried out at pCa 4.0.

Two general protocols were used in experiments with the catalytic subunit of PKA. In the first protocol (protocol 1), a coverslip containing the single myocyte was washed with RS, briefly exposed to RS with 0.1% Triton X-100 for 30–60 s, and then washed three times with standard RS. Phosphorylation was achieved by exposing myocytes to a RS with dithiothreitol (DTT, 6 mg/ml) plus 1 U/μl PKA (catalytic subunit) for ∼25 min (range 15–60 min) at 25°C. Single myocytes were then attached to the recording apparatus as described above, and the tension-pCa relation was determined. In the second protocol (protocol 2), which was used more extensively in these studies, single myocytes were first attached to the recording apparatus, and the membrane was permeabilized as above. The tension-pCa relationship was determined at pH 7.0 and 6.2, the myocyte was exposed to RS containing PKA (see protocol 1) for 30 min, and then the tension-pCa relation at each pH was measured again. In this protocol, the myocyte serves as its own control (i.e., +/−PKA). In control myocytes, PKA was omitted from the RS.
with DTT to serve as control experiments for each protocol. The RS with DTT and PKA was kept for up to 48 h at 4°C before there was degradation of catalytic subunit activity.

The Marquardt-Levenberg nonlinear least-squares fitting algorithm was used to fit the data to the Hill equation

$$P = \frac{[\text{Ca}^{2+}]^{n_H}}{K_{n_H} + [\text{Ca}^{2+}]^{n_H}}$$

where $P$ is the fraction of maximal tension ($P_o$), $n_H$ is the Hill coefficient, and $K$ represents the $p$Ca50 ($-\log[\text{Ca}^{2+}]$), where tension is half-maximal.

Statistics

Values for each group are expressed as means ± SE. ANOVA was used to test for significant differences between groups, with a post hoc Student-Newman-Keuls multiple comparison test to determine significance ($P < 0.05$).

RESULTS

Contribution of TnI to PKA-Mediated Decreases in Myofilament $\text{Ca}^{2+}$ Sensitivity of Tension in Adult Rat Cardiac Myocytes

Investigation of the TnI domains involved in mediating PKA-induced decreases in myofilament $\text{Ca}^{2+}$ sensitivity requires the retention of an intact PKA signaling pathway in primary cultures of adult myocytes, particularly in relation to PKA-mediated changes in $\text{Ca}^{2+}$-activated tension. Previously, it has been shown that the thick filament protein, myosin-binding protein C (MyBP-C), is phosphorylated along with cTnI in response to PKA activation (18). Comparable results are observed in adult cardiac myocytes maintained in culture for 6 days, as seen by the phosphorylation of cTnI and MyBP-C by the catalytic subunit of PKA in membrane-permeabilized adult myocytes (Fig. 2) and by isoproterenol stimulation of intact myocytes (Fig. 3). In freshly isolated cardiac myocytes, PKA activation decreases myofilament $\text{Ca}^{2+}$ sensitivity of tension (15), and this decrease in the $\text{Ca}^{2+}$ sensitivity of myofilament tension is retained in myocytes maintained in culture for 6 days (Fig. 4A). Taken together, the functional and phosphorylation results provide strong evidence that the PKA signaling pathway is intact in cultured adult cardiac myocytes maintained in serum-free culture conditions.
The next step toward determining the TnI domain(s) involved in PKA-mediated changes in myofilament function was to specifically replace endogenous cTnI with a nonphosphorylatable, exogenous TnI within 6 days after TnI gene transfer. Western blot analysis demonstrated that ssTnI, which lacks the Ser-23/Ser-24 PKA phosphorylation sites (1), is expressed in myocytes after gene transfer with a corresponding decrease in endogenous cTnI expression (Fig. 5), in agreement with earlier findings (38). Previous studies also have directly demonstrated that expressed exogenous ssTnI is incorporated in the myofilaments of adult cardiac myocytes without detected changes in contractile protein stoichiometry or in the architecture of the sarcomere (38), thereby demonstrating the specificity of this approach. Thus, observed changes in myofilament function are the direct result of the newly expressed and incorporated ssTnI protein.

The pattern of myofilament protein phosphorylation produced in response to the catalytic subunit of PKA was then studied 6 days after ssTnI gene transfer in membrane-permeabilized myocytes. Both cTnI and MyBP-C were phosphorylated in untreated and AdCMVcTnI-treated myocytes, whereas only MyBP-C was phosphorylated in myocytes expressing the ssTnI isoform (Fig. 2). Similar results were obtained in intact myocytes treated with 100 nM isoproterenol (Fig. 3). Membrane-permeabilized myocytes expressing cTnI or ssTnI were then used to determine the relative contribution of cTnI and MyBP-C phosphorylation (Fig. 2 and Table 1) to PKA-mediated changes in Ca\(^{2+}\)-activated tension. Myofilament Ca\(^{2+}\) sensitivity of tension did not change significantly after PKA treatment in myocytes expressing ssTnI, whereas PKA caused the anticipated decrease in Ca\(^{2+}\) sensitivity in myocytes expressing cTnI (Figs. 4 and 6). This PKA response is TnI isoform specific and is not influenced by adenoviral gene transfer per se, as demonstrated by the comparable PKA-mediated shifts in pCa\(_{50}\) observed in “control” myocytes and AdCMVcTnI-treated myocytes (Fig. 6).

Separate experiments with the nonphosphorylatable TnI chimera, N-slow/card-C TnI, also were carried out with the expectation that the findings would be similar to those observed in ssTnI-expressing myocytes. As with ssTnI, this chimera was expressed in myocytes after gene transfer with a corresponding decrease in
Table 1. Phosphorylation ratio for total TnI/phospholamban 30 min after addition of the catalytic subunit of PKA

<table>
<thead>
<tr>
<th>Myocyte Group</th>
<th>Total TnI/PLB Phosphorylation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.96 ± 0.31</td>
</tr>
<tr>
<td>AdCMVcTnI</td>
<td>1.92 ± 0.32</td>
</tr>
<tr>
<td>AdCMVssTnI</td>
<td>0.29 ± 0.09*</td>
</tr>
<tr>
<td>AdCMVN-slow/card-C TnI</td>
<td>0.32 ± 0.08*</td>
</tr>
<tr>
<td>AdCMV N-card/slow-C TnI</td>
<td>2.69 ± 0.41</td>
</tr>
</tbody>
</table>

Values are means ± SE. Normalized ratios of troponin I (TnI)/phospholamban (PLB) phosphorylation were compared in control (n = 4), AdCMVcTnI-treated (n = 4), AdCMVssTnI-treated (n = 4), AdCMVN-slow/card-C TnI-treated (n = 4), and AdCMV-card/slow-C TnI-treated (n = 4) adult cardiac myocytes maintained in primary culture for 6 days. Phosphorylation of TnI in myocytes expressing slow skeletal (ss) TnI and N-slow/card-C TnI was >20% of the level obtained in control and AdCMVcTnI-treated myocytes. *Experimental vs. control using one-way ANOVA and post hoc Student-Newman-Keuls test (P < 0.05).

cTnI (e.g., amino terminus) plays a significant role in the direction and magnitude of the PKA-induced shift in myofilament Ca\(^{2+}\) sensitivity. In addition, results obtained with the N-card/slow-C TnI chimera indicate that isoform-specific residues in the carboxyl terminal 120 amino acids of ssTnI (e.g., carboxyl terminus) have no significant effect on the magnitude of the tension response to PKA.

Additive Effects of pH and Phosphorylation on Myofilament Ca\(^{2+}\) Sensitivity

The effects of PKA-mediated TnI phosphorylation and acidic pH on myofilament Ca\(^{2+}\) sensitivity of tension were examined as an alternative approach for defining the domain(s) responsible for the phosphorylated TnI-induced decreases in myofilament Ca\(^{2+}\) sensitivity. Acidic pH has been shown to decrease the Ca\(^{2+}\) sensitivity of myofilament tension (28), and recent studies with the N-card/slow-C and N-slow/card-C TnI chimeras provide strong evidence that the carboxyl terminal domain of TnI mediates this response (35, 36). Thus the condition of acidic pH can be introduced after PKA treatment to further determine whether the carboxyl terminus of cTnI influences the magnitude of the phosphorylation-induced decrease in myofilament Ca\(^{2+}\) sensitivity. In myocytes expressing cTnI, the rightward shift in the myofilament Ca\(^{2+}\) sensitivity of tension caused by acidic pH in the presence and absence of phosphorylation was similar in magnitude.
PKA or absence of pretreatment with PKA. In <i>DPcCa50</i> caused by acidic pH (<i>pH</i> 6.2) from <i>pCa50</i> (<i>pH</i> 7.0) in each myocyte in the presence (+PKA) or absence (−PKA) of pretreatment with PKA. In B, <i>ΔpCa50</i> was calculated by subtracting <i>pCa50</i> (+PKA) from <i>pCa50</i> (−PKA) in each myocyte under either normal (<i>pH</i> 7.0) or acidic (<i>pH</i> 6.2) conditions. The <i>ΔpCa50</i> caused by PKA treatment was not significantly affected by acidosis (<i>P</i> > 0.05), and the <i>ΔpCa50</i> caused by acidosis was unaffected by PKA treatment (<i>P</i> > 0.05).

(Fig. 7A). A comparable shift in myofilament Ca<sup>2+</sup> sensitivity also was observed when myocytes previously treated with PKA were exposed to <i>pH</i> 7.0 and acidic <i>pH</i> (Fig. 7B). Thus PKA-induced TnI phosphorylation and acidic pH additively decrease myofilament Ca<sup>2+</sup> sensitivity and appear to act on TnI via independent mechanisms.

**DISCUSSION**

Experiments with adult single cardiac myocytes expressing exogenous TnI proteins provide new evidence that the amino terminal region of TnI is an important domain responsible for the magnitude of the PKA-induced decrease in Ca<sup>2+</sup> sensitivity of myofilament tension. Interestingly, the comparable functional effects of PKA observed with myocytes expressing cTnI or the N-card/slow-C TnI chimera (Fig. 6) demonstrate that isoform differences in the carboxyl terminus of TnI have no effect on the magnitude of the PKA-mediated decrease in myofilament Ca<sup>2+</sup> sensitivity. Instead, these results point to an essential role of the amino portion of cTnI alone, or in combination with non-isoform-specific functional regions in the carboxyl terminus, in mediating PKA-induced alterations in cardiac mechanical function. Additionally, our study provides evidence that PKA-mediated cTnI phosphorylation works independently from acidic pH to shift myofilament Ca<sup>2+</sup> sensitivity. Thus the present findings provide new insight into the specific functional domains within TnI in the intact myofilament of adult cardiac myocytes and further demonstrate the utility of this gene transfer approach for dissecting the functions of individual contractile proteins within adult myocytes.

The contribution of cTnI phosphorylation to PKA-mediated changes in myofilament Ca<sup>2+</sup> sensitivity appears to have important clinical significance. Evidence is accumulating that basal and PKA-mediated TnI phosphorylations change under several conditions known to result in heart failure, including dilated cardiomyopathy (5, 24, 41), ischemic cardiomyopathy (2, 20), and hypertension (22). There is also indirect evidence that β-adrenergic-mediated TnI phosphorylation plays an important role in myocardial relaxation (29, 40). Thus the cardiac response to changes in sympathetic tone may be mediated, in part, by the ability of the TnI to be phosphorylated by PKA.

**Role of TnI Domains in PKA-Mediated Decrease in Myofilament Ca<sup>2+</sup> Sensitivity**

The critical role of phosphorylated TnI in the PKA-mediated decrease in myofilament Ca<sup>2+</sup> sensitivity, along with its potential clinical significance, makes it imperative to understand how TnI transduces the phosphorylation signal into a myofilament response. The phosphorylation event itself is relatively well worked out (reviewed in Ref. 30). Ser-23 and Ser-24 are the primary PKA targets (24, 33); phosphorylation of Ser-24 appears to precede Ser-23 phosphorylation in cTnI (25, 31), and phosphorylation of both serine residues is thought to be necessary for changes in myofilament function (31, 43). There are indications that PKA-induced TnI phosphorylation results in conformational changes within the amino terminal extension (17, 31). However, the molecular events that result from this modification of the amino terminal extension and that ultimately lead to a change in TnI regulation of contractile function remain controversial. Some investigators propose that functional changes caused by PKA phosphorylation are signaled directly through this amino terminal extension (12, 17), whereas others suggest that the Ca<sup>2+</sup>-sensitive region is located in the carboxyl terminus of TnI also may be involved (6, 7). Isoform differences in the carboxyl terminus of TnI influence myofilament Ca<sup>2+</sup> sensitivity of tension (2, 38) and the magnitude of change in myofilament Ca<sup>2+</sup> sensitivity of tension in response to variables such as acidosis (36). These isoform differences do not appear to be involved in determining the magnitude of the Ca<sup>2+</sup>-sensitive region located in the carboxyl terminus of TnI because the magnitude of the response is similar in myocytes expressing N-card/slow-C TnI and cTnI (Fig. 6). This conclusion does not rule out the possibility that the non-isoform-specific, Ca<sup>2+</sup>-sensitive regions within the carboxyl terminus of TnI may contribute to the direction, shape, and magnitude of the PKA-mediated change in myofilament tension-pCa relationship. However, the known regions of TnI that bind other contractile proteins and influence Ca<sup>2+</sup>-sensitive actomyosin ATPase activity in solution studies, including the inhibitory peptide region and secondary actin and TnC binding domains (Tripe et al. (34)), each contain isoform-specific amino acid differences (Murphy et al. (27)). Thus the amino terminus of TnI likely plays a significant role in mediating the conformational changes within TnI in response to PKA, although an as yet undefined non-isoform-specific, Ca<sup>2+</sup>-sensitive domain(s) in the carboxyl portion of TnI also may contribute to this response.
The amino terminus includes the 32-amino acid extension specific for cTnI, which may function as a key domain in mediating the PKA-induced change in myofilament Ca\(^{2+}\) sensitivity in adult myocytes. This amino terminal extension would probably not function as an isolated peptide but would require the presence of a TnI backbone of cTnI or ssTnI origin. Alternatively, the PKA-mediated TnI phosphorylation response could be mediated via a second Ca\(^{2+}\)-sensitive region previously described for the amino terminus of TnI (99 amino acids of N-card/slow-C TnI), which includes the 32-amino acid extension and binding domains for TnC and TnT (reviewed in Refs. 10 and 30). Future experiments with more specifically mutated regions within TnI will be needed to further define the critical interactions within the amino terminus that are necessary to transduce the TnI phosphorylation signal into the shift in myofilament Ca\(^{2+}\) sensitivity of tension.

**Influences of pH and Phosphorylation on Myofilament Ca\(^{2+}\) Sensitivity**

Myofilament tension measurements in myocytes treated with PKA and then exposed to acidic pH provide further evidence that TnI phosphorylation is likely mediated through the amino terminus of the protein. Acidic pH desensitizes myofilaments to Ca\(^{2+}\) via an isoform-specific, carboxyl terminal domain (35, 36). The combined effects of acidosis and PKA-mediated phosphorylation were previously studied in perfused rat hearts, with roughly additive effects observed on developed tension and relaxation (26). Acidosis was imposed during the phosphorylation period, and the additive effects of acidosis and phosphorylation were explained based on acidosis-induced increases in PKA-mediated TnI phosphorylation (26). In the present study, the catalytic subunit of PKA was removed before the introduction of acidic pH to avoid acidosis-induced changes in phosphorylation. The additive decrease in myofilament Ca\(^{2+}\) sensitivity observed with this protocol (e.g., acidosis after TnI phosphorylation; Fig. 7) is evidence that separate functional domains were responsible for acidosis and phosphorylation-mediated decreases in submaximal tension.

**Transduction Model for Phosphorylation-Induced Decreases in Myofilament Ca\(^{2+}\) Sensitivity**

Overall, our results provide new information toward an understanding of the molecular mechanism whereby PKA-mediated TnI phosphorylation leads to a decrease in myofilament Ca\(^{2+}\) sensitivity. Previous investigators have obtained evidence that the phosphorylation of Ser-23/Ser-24 causes the 32-residue amino terminal extension of cTnI to act as a spacer arm that folds upon phosphorylation (1, 7) and increases the separation distance between TnI and TnC (21) such that there is altered Ca\(^{2+}\) binding to regulatory sites on TnC (16). Our results support a mechanism where the amino terminal extension of TnI appears to transduce this phosphorylation signal by interacting directly with TnC, as previously suggested by Al-Hillawi and colleagues (1). Results that support this view include PKA-induced shifts in myofilament Ca\(^{2+}\) sensitivity that are similar in magnitude in myocytes expressing TnI proteins with different isoform-specific carboxyl terminal regions and the additive effects of acidic pH and TnI phosphorylation on myofilament Ca\(^{2+}\) sensitivity of tension. An alternative proposal is that folding of the amino terminus in response to PKA induces a global conformational change in cTnI that is mainly transduced through the carboxyl terminus (6, 7). This mechanism would require the presence of an isoform-independent, Ca\(^{2+}\)-sensitive region within the carboxyl terminus of TnI based on the similar PKA-induced decreases in Ca\(^{2+}\) sensitivity of tension in myocytes expressing cTnI and N-card/slow-C TnI. In the future, it will be important to determine whether phosphorylation of this amino terminal extension acts primarily as a spacer arm to physically decrease TnI-TnC interactions or induces conformational changes within TnC that are then transmitted to the regulatory Ca\(^{2+}\)-binding sites in the amino terminus of TnC.

In summary, experiments presented here establish the importance of the amino terminal region of cTnI in mediating the direction and magnitude of decreased myofilament Ca\(^{2+}\) sensitivity after PKA activation. With the use of gene transfer approaches, hypotheses addressing the relative function of the 32-amino acid extension of cTnI vs. the additional 67 amino terminal residues present in N-card/slow-C TnI during PKA activation can now be designed to gain more knowledge about the function of TnI during this signaling process within the context of the intact myofilament.

We appreciate helpful comments from Dan Michele and Philip Wahr on earlier versions of the manuscript.

This work was supported by grants from the National Institutes of Health and American Heart Association to J. M. Metzger. J. M. Metzger is an Established Investigator of the American Heart Association, and M. V. Westfall was the recipient of a Scientist Development grant from the American Heart Association.

**REFERENCES**


6. Chandra M, Dong WJ, Pan BS, Cheung HC, and Solaro RJ. Effects of protein kinase A phosphorylation on signaling between...


