Chimera Analysis of Troponin I Domains That Influence Ca^{2+}-Activated Myofilament Tension in Adult Cardiac Myocytes

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Abstract—The goal of this study was to investigate isoform-specific functional domains of the inhibitory troponin subunit, troponin I (TnI), as it functions within the intact myofilaments of adult cardiac myocytes. Adenovirus-mediated gene transfer was used to deliver and express a TnI chimera composed of the amino terminus of cardiac TnI (cTnI) and the carboxy terminus of slow skeletal TnI (ssTnI) in adult rat cardiac myocytes. The TnI chimera, designated N-card/slow-C TnI, was expressed and incorporated into myofilaments after gene transfer, without detectable changes in contractile protein stoichiometry or sarcomere architecture. Interestingly, force at submaximal Ca^{2+} levels was markedly elevated in single permeabilized myocytes expressing the N-card/slow-C TnI chimera relative to force generated in adult myocytes expressing ssTnI or cTnI. Based on these results, a hierarchy of myofilament Ca^{2+} sensitivity is emerging by use of TnI chimera analysis, with the order of sensitivity being N-card/slow-C TnI >> ssTnI >> cTnI. These results also strongly suggest that independent isoform-specific domains in both the amino and carboxy portions of TnI influence myofilament Ca^{2+} sensitivity. In additional studies carried out under pathophysiological ionic conditions (pH 6.2), the dramatic acidosis-induced decrease in myofilament Ca^{2+} sensitivity observed in myocytes expressing cTnI was blunted in myocytes expressing N-card/slow-C TnI in a manner similar to that in ssTnI-expressing myocytes. These results demonstrate that there is a pH-sensitive domain residing in the carboxy-terminal portion of TnI. The dissection of isoform-specific functional domains under physiological and acidic pH conditions demonstrates the utility of TnI chimeras for analysis of TnI function and provides important insights into the overall function of TnI within the intact myofilament of adult cardiac myocytes. (Circ Res. 2000;86:470-477.)

Key Words: troponin I ■ chimera ■ myofilaments ■ heart ■ gene transfer

Troponin is a multisubunit protein critical for regulating Ca^{2+}-activated tension in striated muscle. The inhibitory troponin subunit, troponin I (TnI), acts to inhibit tension development until Ca^{2+} binds to regulatory sites on troponin C (TnC). In the absence of a high-resolution structure for TnI and controversy about lower resolution structures, it remains unknown whether TnI acts directly as a molecular switch through its interaction with actin or acts indirectly as a facilitator of inhibition via interactions with other thin-filament proteins. Most information about the molecular switch properties of TnI comes from biochemical studies using fragments or truncated TnI proteins and results from these studies have defined the thin-filament proteins that interact with specific regions within the primary structure of TnI in a Ca^{2+}-sensitive manner. However, it has remained difficult to investigate the function of the full-length protein in the context of an intact myofilament.

Recent progress has been made toward more precisely defining the role of the TnI subunit as it functions in the myofilament of individual cells by the use of virus-mediated gene transfer into adult cardiac myocytes. This approach has directly demonstrated that there are TnI isoform–specific influences on myofilament function by comparing myocytes expressing the endogenous cardiac isoform with myocytes expressing slow skeletal TnI (ssTnI), an isoform expressed during fetal cardiac development. Rapid and efficient exchange of endogenous cardiac TnI (cTnI) by exogenous ssTnI is observed within the myofilaments of the myocyte by use of this approach. Therefore, TnI isoform–specific influences on myofilament function have been defined. Recently, the functional results with viral gene transfer have been supported in transgenic mice expressing ssTnI within the myocardium.

To better understand how TnI acts to inhibit Ca^{2+}-activated tension, it is necessary to elucidate the function of individual TnI domains within the intact myofilament. Previous investigations using biochemical approaches have concluded that the carboxy terminus of TnI contains the major region influencing Ca^{2+} sensitivity. In contrast, a recent study in...
cardiac myocytes expressing a chimeric TnI protein, composed of the amino terminus of ssTnI and the carboxy terminus of cTnI (now designated N-slow/card-C TnI), has provided evidence for the presence of at least 2 domains within TnI that have isoform-specific effects on myofilament Ca\(^{2+}\) sensitivity.\(^{11}\) The conflicting results obtained with these 2 approaches may be due to the use of TnI fragments in biochemical studies, which may assume a tertiary structure different from that of the intact protein.\(^{12}\) In addition, mechanical constraints placed on TnI and/or the myofilament protein stoichiometry are likely to be much different in solution compared with the intact myofilament. Thus, more experiments are clearly required to determine whether 1 or 2 Ca\(^{2+}\)-sensitive domains operate within the TnI protein as it functions in the intact myofilament of adult cardiac myocytes under physiological and pathophysiological experimental conditions.

A major goal of the present study was to express a TnI chimera in adult cardiac myocytes to provide new information about the putative isoform-specific Ca\(^{2+}\)-sensitive domains. The new TnI chimera tested here, designated N-card/slow-C TnI, consists of the amino terminus of cTnI and the carboxy terminus of ssTnI. Myofilament Ca\(^{2+}\) sensitivity should be the same in adult cardiac myocytes expressing ssTnI or N-card/slow-C TnI if only the carboxy terminus influences the TnI protein as it functions in the intact myofilament of adult cardiac myocytes under physiological and pathophysiological experimental conditions.

Materials and Methods

**Mutagenesis Strategy**

Full-length rat wild-type ssTnI and cTnI cDNAs (kind gifts of A. Murphy, Johns Hopkins University, Baltimore, Md; see Figure 1) were used to generate the N-card/slow-C TnI chimera. To begin construction of the chimeric TnI, cTnI was mutagenized to form cTnI-SacI, as described by Westfall et al.\(^{11}\) A 390-bp EcoRI-SacI fragment isolated from pGEM-3ZcTnI-SacI was ligated with an EcoRI-SacI digest of pGEM-3ZssTnI to form the pGEM3ZN-card/slow-C TnI. The complete nucleotide sequence of N-card/slow-C TnI was confirmed by DNA sequencing, and N-card/slow-C TnI was subcloned into an adenovirus shuttle vector plasmid by using EcoRI and BamHI sites to form pAdCMVN-card/slow-C TnI.\(^{11,14}\) Chimeric TnI protein production was verified in HEK 293 cells before producing the adenovirus.\(^{11}\)

**Generation of Adenovirus Vectors**

Recombinant adenovirus vectors were constructed by cotransfecting shuttle plasmids containing TnI cDNAs (cTnI, ssTnI, and N-card/slow-C TnI) and pJM17 into HEK 293 cells, as described in detail previously.\(^{14}\)

**Primary Cultures of Rat Ventricular Myocytes**

Ventricular myocytes were isolated from adult female rats as described in detail by Westfall et al.\(^{14}\)

**Analysis of Protein Composition by Gel Electrophoresis and Western Blots**

**Gel Electrophoresis**

Approximately 10 ventricular myocytes were collected on the tip of a glass micropipette and transferred to microcentrifuge tubes containing 10 \(\muL\) of sample buffer for analysis by gel electrophoresis.\(^{9,15}\) Fiber segments of soleus muscles were collected as described previously.\(^{16}\) Samples were sonicated for 10 minutes and briefly centrifuged before analysis by gel electrophoresis. Gels for SDS-PAGE were prepared and stained as described previously.\(^{15,17}\) Stained gels were scanned and analyzed with Multi-Analyst software (Bio-Rad).
Western Blot Analysis
Cultured ventricular myocytes from coverslips were collected in sample buffer 4 to 7 days after plating and gene transfer, separated by gel electrophoresis as described above, and then transblotted onto polyvinylidene fluoride membrane as previously described in detail.17 Protein expression in HEK 293 cells was identified by use of a similar protocol. Permeabilized myocytes were prepared by transferring cells to relaxing solution (see composition below) containing 0.1% Triton X-100 for 1 minute; the cells were then rinsed 3 times in relaxing solution alone before collection in sample buffer. After proteins were separated by SDS-PAGE, immunodetection was carried out as described by Westfall et al.17

Indirect Immunohistochemistry in Single Cardiac Myocytes
Indirect immunofluorescence with a dual monoclonal antibody (mAb) protocol14,17 was used to determine the extent of thin-filament remodeling resulting from ectopic N-card/slow-C TnI expression within single cardiac myocytes in primary culture. The primary mAbs used for this assay were MAB 1691 (Chemicon) and TI-1 (kind gift of S. Schiaffino, University of Padua, Italy).18

Measurement of Ca$^{2+}$-Activated Tension in Single Cardiac Myocytes at pH 7.0 and pH 6.2
Complete details of the relaxing and activating solutions used, of the experimental chamber, and of the attachment procedure for mounting single rod-shaped cardiac myocytes have been reported elsewhere.16 Ca$^{2+}$-activated tension was measured in single myocytes, as described in detail elsewhere.16

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

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Expression and Myofilament Incorporation of N-Card/Slow-C TnI in Adult Cardiac Myocytes
Initial experiments were designed to determine whether the chimera protein, N-card/slow-C TnI, is expressed and incorporated into the myofilament after viral delivery into adult cardiac myocytes. Expression of N-card/slow-C TnI increased over time, as assessed by Western blots (Figures 2A and 2B), and the chimera made up ~90% of total TnI by 6 days postgene transfer (Figure 2B). Treatment of myocytes with AdCMVssTnI resulted in similar expression levels of ssTnI, and there was a corresponding decrease in cTnI expression in the myocytes expressing either ssTnI or N-card/ slow-C TnI (Figure 2A). This pattern of exogenous TnI expression is consistent with the 3.2-day half-life of TnI previously observed by Martin.19

Further analysis of Western blots indicated that total TnI protein was not changed in myocytes expressing ssTnI or N-card/slow-C TnI compared with control values when normalized to troponin T (TnT), tropomyosin (Tm), or a silver-stained band on the accompanying gel (Figure 2A, Table 1). Myosin and myosin light chain stoichiometry also were not affected by gene transfer (Table 2). The isoform compositions of TnT, Tm (Figure 2A), myosin, and the myosin light chains (results not shown) were also unchanged from the control isoforms in myocytes expressing ssTnI or N-card/slow-C TnI.

These results confirm previous results9,11 showing that exogenous TnI protein expression does not alter TnI stoichiometry within the thin filament and does not change the isoform expression pattern of other contractile proteins in the myofilament of adult cardiac myocytes; the results also collectively demonstrate the specificity of this approach.

Western blot analysis and indirect immunohistochemistry were then used to determine whether the exogenous TnI expressed in cardiac myocytes was incorporated into the
TABLE 1. Stoichiometry of Contractile Proteins Determined From Western Blots and Silver-Stained Portion of Each SDS-PAGE Gel

<table>
<thead>
<tr>
<th>Myocyte Treatment</th>
<th>TnI/Tm</th>
<th>TnI/TnT</th>
<th>TnI/Silver Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCMVcTnl</td>
<td>1.07±0.13</td>
<td>0.83±0.04</td>
<td>1.26±0.24</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=9)</td>
</tr>
<tr>
<td>AdCMVssTnl</td>
<td>1.34±0.14</td>
<td>1.13±0.08</td>
<td>1.04±0.13</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=5)</td>
<td>(n=9)</td>
</tr>
<tr>
<td>AdCMVN-card/slow-C TnI</td>
<td>0.75±0.10</td>
<td>0.92±0.11</td>
<td>1.17±0.13</td>
</tr>
<tr>
<td>TnI chimera</td>
<td>(n=24)</td>
<td>(n=22)</td>
<td>(n=38)</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were compared by 1-way ANOVA; n indicates the number of lanes analyzed on Western blots. Ratios were compared with control values (n=11 to 14), which were set to 1.0. There were no significant differences (P>0.05) compared with control values or between groups.

TABLE 2. Stoichiometry of MHC and MLCs Determined by SDS-PAGE

<table>
<thead>
<tr>
<th>Myocyte Treatment</th>
<th>Control</th>
<th>AdCMVN-Card/Slow-C TnI Chimera</th>
</tr>
</thead>
<tbody>
<tr>
<td>aMHC/total MHC</td>
<td>0.84±0.08</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
<tr>
<td>MLC/total MLC</td>
<td>0.43±0.06</td>
<td>0.37±0.04</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

Values are mean±SEM and were compared by Student’s t test. MHC indicates myosin heavy chain; MLC, myosin light chain; and n, number of gel samples analyzed. There were no significant differences among groups (P>0.05).

myofilament. One approach for examining myofilament incorporation was to compare exogenous TnI expression on Western blots from intact and membrane-permeabilized cardiac myocytes expressing N-card/slow-C TnI. Both the pattern of exogenous N-card/slow-C TnI expression over time (Figure 3A) and the overall proportion of exogenous TnI (Figure 3B) were the same in intact and permeabilized myocytes. Myofilament incorporation of exogenous TnI was also examined by using indirect immunohistochemical labeling of adult cardiac myocytes. In these experiments (Figure 4), the non–isoform-specific anti-TnI mAb, MAB 1691, recognized TnI in control and AdCMVN-card/slow-C TnI–treated cardiac myocytes at all time points. A striated pattern of immunolabeling was observed across the length, width, and depth of the cells in both groups of myocytes (Figures 4A and 4C) and in myocytes treated with AdCMVcTnl (results not shown). All control (Figure 4B) and AdCMVcTnl–treated myocytes also showed positive immunostaining with the TI-1 mAb, whereas the proportion of AdCMVN-card/slow-C TnI–treated myocytes that stained positive with the cardiac-specific TI-1 mAb decreased from 75% after 4 days (n=543) to 49% (n=507), 22% (n=649), and 11% (n=526) after 5, 6 (see Figure 4D), and 7 days in primary culture, respectively. Taken together, the Western blot analysis and indirect immunolabeling results indicate that N-card/slow-C TnI is expressed and accurately incorporated into the sarcomere of adult cardiac myocytes without detectable pools of protein accumulating in the cytosol. Collectively, these results confirm that gene transfer results in the specific replacement of endogenous cTnl with exogenous TnI in the thin filament. This specific replacement of cTnl by N-card/slow-C TnI in the myofilament indicates that changes in myofilament function are directly attributed to the effects of exogenous TnI on contractile function (see below).

Functional Effects of N-Card/Slow-C TnI on Ca2+-Activated Tension in Adult Cardiac Myocytes

Myofilament function in adult single cardiac myocytes expressing N-card/slow-C TnI was examined to investigate isoform-specific Ca2+-sensitive domains within TnI. Fast time-based recordings show that steady-state isometric tension was increased at intermediate Ca2+ concentrations in myocytes expressing N-card/slow-C TnI (Figure 5A), such that the tension-pCa relation in myocytes expressing N-card/slow-C TnI was shifted significantly leftward relative to the relation observed in myocytes expressing cTnl (Figure 5B) or...
scientific (nH) were not significantly different among myocytes in myofilament Ca$^{2+}$ the TnI domain responsible for this influence of pH on expressing cTnI, ssTnI, and N-card/slow-C TnI to investigate a measure of myofilament Ca$^{2+}$ levels was similar to that observed in myocytes expressing ssTnI (Figure 5C). This leftward shift is reflected in the pCa50, which recognizes all isoforms of TnI and was detected with goat anti-mouse antibody conjugated to FITC (1:200). In panels B and D, the cTnI-specific mAb, TI-1 (1:1000),18 was used, and immunolabeling was detected with goat anti-mouse antibody conjugated to Texas Red (1:100). The immunolabeling observed in control myocytes was also observed in myocytes treated with AdCMVcTnl (results not shown). Immunolabeling with the TI-1 mAb was not detectable by immunofluorescence (D) or by Western blot in myocytes expressing N-card/slow-C TnI (1:1000). The striated immunolabeling pattern is demonstrated in the inset for each image. The striated image obtained with the MAB 1691 anti-Tnl mAb in panel C indicates that N-card/slow-C TnI is expressed and incorporated into the myofilament. Bar=10 μm (applies to all panels). Inset images represent ~8 μm.

ssTnI (Figure 5C). This leftward shift is reflected in the pCa50, a measure of myofilament Ca$^{2+}$ sensitivity, which was significantly increased in myocytes expressing N-card/slow-C TnI compared with values in control and ssTnI-expressing myocytes (Figure 5D). Further analysis of tension in myocytes expressing N-card/slow-C TnI indicated that the threshold [Ca$^{2+}$] required for tension development above baseline levels was similar to that observed in myocytes expressing ssTnI (results not shown) and that this threshold was significantly lower than that observed in control myocytes. Finally, resting tension, maximum tension (Po), and the Hill coefficient (nH) were not significantly different among myocytes expressing cTnl, ssTnI, and N-card/slow-C TnI (see Figure 5 legend).

Effects of Acidic pH on Ca$^{2+}$-Activated Tension in Myocytes Expressing N-Card/Slow-C TnI

TnI has an isoform-specific influence on myofilament Ca$^{2+}$ sensitivity when acidic pH develops within cardiac myocytes.9,11 Submaximal Ca$^{2+}$-activated tension measurements were made at pH 7.0 and pH 6.2 in permeabilized myocytes expressing cTnl, ssTnI, and N-card/slow-C TnI to investigate the Tnl domain responsible for this influence of pH on Ca$^{2+}$-sensitive tension development. As expected, the change in myofilament Ca$^{2+}$ sensitivity detected as the difference between the pCa50 at pH 7.0 and pCa50 at 6.20 was greatest in myocytes expressing cTnl, and this pH-induced shift was significantly blunted in myocytes expressing ssTnI (Figure 6). Interestingly, the acidosis-induced shift in myofilament Ca$^{2+}$ sensitivity was also blunted in myocytes expressing the N-card/slow-C TnI chimera (Figure 6) and was similar to the response observed in myocytes expressing the ssTnI isoform. Further analysis of the contractile response to acidosis in myocytes expressing either ssTnI or N-card/slow-C TnI indicated that maximum tension also decreased to a lesser extent than in control myocytes when activating solution pH decreased from 7.0 to 6.2 (see legends of Figures 5 and 6). The Hill coefficients at acidic pH were not significantly different in cardiac myocytes expressing cTnl, ssTnI, and N-card/slow-C TnI. This ability of TnI isoform expression to influence pH-induced changes in maximum tension and myofilament Ca$^{2+}$ sensitivity of tension indicates that TnI serves as an important pH sensor within the myofilaments. These results also demonstrate that ssTnI and the N-card/slow-C TnI chimera share a less sensitive isoform-specific pH-sensitive domain.

Discussion

An important new finding of the present study is that the expression and incorporation of N-card/slow-C TnI into the myofilaments of adult cardiac myocytes result in a marked increase in submaximal Ca$^{2+}$-sensitive tension compared with the tension in adult cardiac myocytes expressing either cTnl or ssTnI. This finding, plus studies with the N-slow/card-C TnI chimera,1 suggests that there is a hierarchy of myofilament Ca$^{2+}$ sensitivity in adult myocytes expressing different TnI isoforms/chimeras. Expression of N-card/slow-C TnI produces the highest Ca$^{2+}$ sensitivity of tension in myofilaments of adult cardiac myocytes, followed by the expression of ssTnI, cTnl, and N-slow/card-C TnI (see Reference 11). The emergence of this hierarchy in adult cardiac myocytes expressing TnI chimeras and isoforms provides strong support for the presence of 2 distinct regions within TnI that influence myofilament Ca$^{2+}$ sensitivity. Results of the present study also demonstrate that the myofilament response to acidic pH in adult myocytes expressing N-card/slow-C TnI matched the shift observed with the ssTnI isoform. This result, together with earlier work on N-slow/card-C TnI,11 offers compelling evidence that pH-induced changes in myofilament Ca$^{2+}$ sensitivity lie in the carboxy terminus of the TnI protein. In future studies, it will be interesting to determine the influence of these TnI chimeras on intact myocyte function to more fully understand the role of TnI in contractile function and to further pinpoint, if possible, more exact domains within TnI that are important for Ca$^{2+}$ sensitivity.

Two Isoform-Specific Ca$^{2+}$-Sensitive Regions Within TnI Identified by Chimera Analysis

Two isoform-specific Ca$^{2+}$-sensitive domains are essential to explain the hierarchy of myofilament Ca$^{2+}$ sensitivity presented here (Figure 5). We propose that 1 of the isoform-specific domains resides in the carboxy portion of TnI, with a second isoform-specific region in the amino terminus of TnI. This idea differs from prior concepts derived from...
biochemical studies in which the primary Ca\textsuperscript{2+}-sensitive region was postulated to lie in the carboxy region of TnI, with the amino terminus having no influence on Ca\textsuperscript{2+} sensitivity. 1,7,8 However, the view that there are 2 isoform-specific domains is supported by recent work with the N-slow/card-C TnI chimera,11 which displayed decreased myofilament Ca\textsuperscript{2+} sensitivity compared with myocytes expressing either TnI isoform. In the carboxy region, cTnI has a less Ca\textsuperscript{2+}-sensitive effect than ssTnI, inasmuch as myocytes expressing cTnI or N-slow/card-C TnI share a common carboxy terminus and exhibit decreased myofilament Ca\textsuperscript{2+} sensitivity relative to ssTnI-expressing myocytes. 11 Results from the present study demonstrate that the shared carboxy terminus in ssTnI and N-slow/card-C TnI chimera causes heightened myofilament Ca\textsuperscript{2+} sensitivity relative to cTnI (Figure 5). Although the exact amino acid sequence(s) contributing to isoform-specific myofilament Ca\textsuperscript{2+} sensitivity remains to be determined, evidence from biochemical studies7,8 indicates that the carboxy portion of TnI contains 2 Ca\textsuperscript{2+}-sensitive TnC binding sites and 2 actin binding sites and that 1 of these amino acid sequences may influence the Ca\textsuperscript{2+} sensitivity of myofilament tension.

In surprising contrast to the carboxy region, the amino portion of cTnI appears to produce greater Ca\textsuperscript{2+} sensitivity than the corresponding amino-terminus region of ssTnI. This interpretation is based on the increased myofilament Ca\textsuperscript{2+} sensitivity observed in myocytes expressing N-card/slow-C TnI compared with ssTnI (Figure 5) and the decreased myofilament Ca\textsuperscript{2+} sensitivity observed in myocytes expressing N-slow/card-C compared with myocytes expressing cTnI.11 Thus, the region of the amino terminus responsible for influencing myofilament Ca\textsuperscript{2+} sensitivity is TnI isoform dependent. The newly defined isoform-specific Ca\textsuperscript{2+}-sensitive amino-terminus TnI domain has not been revealed

Figure 5. Tension-pCa relations in permeabilized adult ventricular myocytes cultured for 5 to 6 days after gene transfer of ssTnI or N-card/slow-C TnI and in control myocytes. A, Original fast time-based recordings of Ca\textsuperscript{2+}-activated isometric tension development in a control (a through d) and an AdCMVN-card/slow-C TnI–treated (e through h) single myocyte maintained in primary culture for 5 to 6 days. The pCa values of the activating solutions are 4.0 (a, d, e, and h), 6.2 (b and g), 5.7 (c), and 7.0 (f), respectively (vertical scale bar is 5.5 kN/m\textsuperscript{2}). Active tension was obtained by subtracting resting tension in relaxing solution (pCa 9.0) from total tension (see difference between arrows in recording a) at each pCa. The P\textsubscript{o} was 19.8 kN/m\textsuperscript{2} for the control myocyte and 16.0 kN/m\textsuperscript{2} for the myocyte expressing the chimera. B, Comparison of tension-pCa relation in control (n = 6, ●) and N-card/slow-C TnI–expressing (n = 8, ▲) permeabilized adult ventricular myocytes. C, Comparison of tension-pCa relation in control (n = 6, ●) and ssTnI-expressing (n = 4, ○) permeabilized adult ventricular myocytes. Myocytes were maintained in primary culture for 5 to 6 days. Cooperativity (control n = 1.95 ± 0.23, AdCMVssTnI n = 1.15 ± 0.17, and AdCMVN-card/slow-C TnI n = 1.64 ± 0.22) and maximum tension (control P\textsubscript{o} 13.13 ± 2.29 kN/m\textsuperscript{2}, AdCMVssTnI P\textsubscript{o} 11.13 ± 2.27 kN/m\textsuperscript{2}, and AdCMVN-card/slow-C TnI P\textsubscript{o} 13.99 ± 1.71 kN/m\textsuperscript{2}) were not significantly different (P > 0.05) among the 3 groups, as determined by 1-way ANOVA. D, Comparison of the pCa\textsubscript{50} in control myocytes, myocytes treated with AdCMVssTnI, and myocytes treated with AdCMVN-card/slow-C TnI. The pCa\textsubscript{50} increased in treated myocytes expressing ssTnI relative to control values. *P < 0.05 vs control; †P < 0.05 in myocytes expressing N-card/slow-C TnI vs myocytes expressing ssTnI. Statistical comparisons were carried out by 1-way ANOVA and post hoc Newman-Keuls test.
in earlier biochemical studies. Work with amino-terminus fragments has shown that this region of cTnI binds in a largely Ca$^{2+}$-independent antiparallel fashion to the carboxy terminus of TnC, which contains the structural Mg$^{2+}$/Ca$^{2+}$ binding sites. The cTnI amino acids from 54 to 79, which are included in the amino-terminus region described in the present study, along with comparable regions within other TnI isoforms also appear to bind to TnT. Thus, isoform-specific changes in myofilament Ca$^{2+}$ sensitivity that are mediated by the amino-terminus region of TnI (Figure 5) may result from interactions of this domain with TnT and/or TnC. Further localization of the pH domain of TnI is the region responsible for the isoform-specific changes in myofilament Ca$^{2+}$ sensitivity observed in response to acidic pH. The pH-induced decrease in myofilament Ca$^{2+}$ sensitivity observed in adult cardiac myocytes expressing cTnI was blunted to a similar extent in myocytes expressing ssTnI or N-card/slow-C TnI (Figure 6), which share the same carboxy-terminus sequence. Taken together, our work with TnI chimeras conclusively demonstrates that this isoform-specific pH-sensitive domain is restricted to the carboxy domain of TnI. Further localization of the pH domain may be of clinical importance in view of the fact that this domain may play a prominent role in mediating the myocardial response to acidosis that accompanies ischemia.

Acidosis-mediated changes in maximum tension also were attenuated in adult cardiac myocytes expressing ssTnI or N-card/slow-C TnI (Figure 6 legend). Although TnI has not previously been considered to play a role in the maximum tension changes brought on by acidic pH, a similar trend previously was observed in myocytes expressing ssTnI without statistical significance. In addition, earlier comparative studies have shown that the magnitude of acidosis-induced changes in maximum tension are muscle-type dependent and that there are differences in TnI isoforms among these muscles. Actomyosin ATPase activity and stiffness also decrease in cardiac myofilaments with acidosis, but to a lesser extent than maximum tension. Thus, multiple processes likely operate during acidosis-induced changes in maximum tension, including decreases in crossbridge cycling, the number of force-generating crossbridges, and the force per crossbridge. We postulate that isoform-specific effects of TnI on the maximum tension response to acidic pH may be due to TnI influences on a number of these variables.

Figure 6. Effect of acidic pH on the calculated difference between the pCa$^{50}$ at pH 7.0 and pH 6.2 (ΔpCa$^{50}$) for cTnI-expressing control myocytes (n=6), myocytes treated with AdCMVssTnI (n=4), and myocytes treated with AdCMV N-card/slow-C TnI (n=7). Results were compared by 1-way ANOVA and post hoc Student-Newman Keuls test; *P<0.05 was considered significant vs control. Maximum tension (P) at pH 6.20 normalized to P at pH 7.0 was significantly different vs control (control P/Po 0.51±0.03, AdCMVssTnI P/Po 0.70±0.04 [P<0.05], and AdCMV N-card/slow-C TnI P/Po 0.67±0.04 [P<0.05]). However, cooperativity at pH 6.20 was not significantly different (P>0.05) among the 3 groups of myocytes (results not shown).

N-Card/Slow-C TnI Chimera May Influence the Thin-Filament Regulatory State

The mechanism whereby myocytes expressing N-card/slow-C TnI enhance submaximal myofilament tension is not known but may be due to a direct effect on Ca$^{2+}$ binding to TnC. Alternatively, the chimera could influence the proportion of thin-filament units residing in different regulatory states. In the 3-state model of thin-filament regulation, the blocked state prevents interactions between actin and myosin, the closed state allows weak interactions between myosin and actin, and in the open state, strong force-generating interactions develop between myosin and actin. An increased proportion of thin filament units may occupy the closed rather than blocked state in myocytes expressing N-card/slow-C TnI compared with myocytes expressing ssTnI or cTnI. A greater proportion of thin-filament units in the closed state versus the blocked state would increase the probability of strong force-generating interactions between actin and myosin in the presence of submaximal Ca$^{2+}$ levels, and the higher tension observed in myocytes expressing N-card/slow-C TnI compared with cTnI or ssTnI-expressing myocytes (Figure 5) would be expected. There is also the possibility that N-card/slow-C TnI acts to heighten submaximal tension by enhancing strong crossbridge-mediated activation of the thin filament.
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