Force–pCa Relation and Troponin T Isoforms of Rabbit Myocardium

Rashid Nassar, Nadia N. Malouf, Michael B. Kelly, Annette E. Oakeley, and Page A.W. Anderson

We have previously reported the existence of at least four troponin T isoforms in rabbit ventricular muscle and described the changes in their distribution with development. In this report we test whether the proportions of the troponin T isoforms are related to the sensitivity of the myofilaments to calcium. We measured the force–pCa relations in 12 detergent-skinned ventricular strands of cardiac muscle from newborn (2–5-day-old) rabbits. We determined from each strand the amount of each troponin T isoform relative to the total amount of troponin T by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and densitometric scans of Western blots probed with a cardiac-specific troponin T monoclonal antibody, MAb 13-11. To assess the presence of different relative amounts of cardiac and slow skeletal troponin I among the strands, we determined the amount of cardiac troponin I relative to tropomyosin. We determined the Hill coefficient and the pCa for half-maximal force, pCa50, for each strand. pCa50 was related directly to the relative amount of troponin T2 (p slope = 0.037). Our results do not indicate a relation between the Hill coefficient and troponin T2. We also did not find a relation between pCa50 and the cardiac troponin I/tropomyosin ratio, which suggests that the correlation between pCa50 and troponin T2 was not a result of changes in the relative amounts of cardiac and slow skeletal muscle troponin I. Our findings indicate that a relation exists between the force–pCa characteristics of rabbit myocardium and the troponin T isoforms that it expresses, suggesting a role for troponin T in modulating the sensitivity of cardiac myofilaments to calcium. (Circulation Research 1991;69:1470–1475)

Troponin T, I, and C are components of the thin filament regulatory system that control cardiac myofilament contractile response to calcium.1–3 Troponin I inhibits thick and thin filament interaction; this inhibition is suppressed by the binding of calcium to troponin C. Troponin T binds the troponin complex to tropomyosin. Although the functional roles of troponin I and troponin C have been studied extensively (e.g., References 4–7), the role of troponin T in modulating the sensitivity of the cardiac myofilaments to calcium is not known. That troponin T is functionally important is strongly suggested by studies of others. Tobacman and Lee8 found (in a reconstituted system of contractile proteins) that myofibrillar ATPase activity is affected by bovine cardiac troponin T isoforms. Schachat et al9 found a relation between force–pCa characteristics of individual fast skeletal muscle fibers and their troponin T and tropomyosin isoforms.

A useful approach for finding out how troponin T affects the response of cardiac myofilaments to calcium is to examine the response to calcium of myocardial preparations that contain different troponin T isoforms. Rat, chicken, rabbit, and dog myocardium are potential preparations: two or more cardiac isoforms are expressed in each of these species in a developmentally regulated manner.10–15 Changes in cardiac troponin T isoform expression have been proposed as the basis for a developmental increase in cardiac myofibril calcium binding,16 for developmental changes in the sensitivity of cardiac myofilaments to calcium,17,18 and for the acquisition of myocardial sensitivity to acidosis.19

In this study we measured force–pCa relations of ventricular strands obtained from neonatal rabbits. We found a correlation between the calcium concentration that produces half-maximal activation (pCa50) and the troponin T isoform composition of the myofibrils. These results suggest that isoforms of troponin T are important in modulating the sensitivity of cardiac myofilaments to calcium.
TABLE 1. The Biophysical and Structural Properties of the Strands

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sarcomere length (µm)</th>
<th>pCa_{50}</th>
<th>Hill coefficient</th>
<th>TnT_{1} (%)</th>
<th>TnT_{2} (%)</th>
<th>TnT_{3} (%)</th>
<th>TnT_{4} (%)</th>
<th>TnI/Tm</th>
<th>Strand diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.1</td>
<td>6.08</td>
<td>2.3</td>
<td>8.1</td>
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<tr>
<td>2</td>
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<td>2.3</td>
<td>8.5</td>
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</tr>
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<td>1.5</td>
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<td>70</td>
</tr>
<tr>
<td>Mean</td>
<td>2.28</td>
<td>5.85</td>
<td>2.5</td>
<td>9.4</td>
<td>42.5</td>
<td>3.7</td>
<td>44.3</td>
<td>0.96</td>
<td>95</td>
</tr>
<tr>
<td>SD</td>
<td>0.13</td>
<td>0.17</td>
<td>0.5</td>
<td>2.9</td>
<td>6.7</td>
<td>1.6</td>
<td>8</td>
<td>0.24</td>
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</table>

TnI, troponin I; Tm, tropomyosin.

**Materials and Methods**

**Preparations**

Free-running ventricular strands (n=12) were obtained from hearts of neonatal New Zealand White rabbits (n=9; mean weight, 56 g; range, 42–69 g) 2–5 days old. Strands were round or flat and ribbonlike in cross section. The major diameter of each preparation is listed in Table 1. After cervical dislocation, the heart was removed and washed in physiological solution ([mM] NaCl 140, KCl 4, MgCl2 1, dextrose 5.6, MOPS 10, and CaCl2 1, pH 7.4). Other solutions used in this study include “relaxing” solution (pCa 9, pMg 3.00, pMgATP 2.50, phosphocreatine 12 mM, creatine phosphokinase 15 units/ml, EGTA 10 mM, and 2% polyvinylpyrrolidone-40, pH 7.1, ionic strength 0.19 M), “skinning solution” (relaxing solution to which 0.5% Triton X-100 had been added), and “activating” solution (which had the same free cation concentrations as relaxing solution but pCa was 4.5). Solutions with pCa ranging from 7.0 to 4.75 were prepared by combining different amounts of relaxing and activating solutions (see Reference 2). Ionic concentrations were calculated using a computer program.20,21

After the heart was washed in physiological solution, it was immersed for 10 minutes in calcium-free physiological solution containing 2 mM EGTA. To remove the membrane systems, the ventricular free walls were incised and the heart was put in skinning solution for 30 minutes at 4°C. The heart was then placed in relaxing solution.

The strand was placed inside the perfusion cuvette, which contained relaxing solution, and attached by small forces at one end to the force transducer22,23 (OPTIM, Scientific Instruments GmbH, Heidelberg, FRG) and at the other to a micrometer screw. The rear port of the cuvette was connected to a multiway valve system that allowed up to 16 different solutions to be applied to the strand rapidly and ensured complete solution replacement. Sarcomere length was measured by passing light from a He-Ne laser through the strand. A microscope objective (x40, 0.75 numerical aperture; Zeiss) and an optical system mounted above the cuvette projected the diffraction pattern onto a calibrated screen. Before the force-pCa data were obtained, the strand was stretched from its slack length while the sarcomere length was monitored by measuring the distance between the first-order maxima of the diffraction pattern. Uniformity of sarcomere length was evaluated along the length of the strand. Discrete first orders were required for the strand to be studied. The force-pCa relations were determined at a sarcomere length of about 2.2 µm (2.0–2.4 µm) at room temperature. Although sarcomere length has been shown to affect pCa_{50} in adult myocardium,24,25 no significant correlation was found between pCa_{50} and sarcomere length over this range in these neonatal preparations.

**Force-pCa Data Acquisition**

The strand was placed at its study length, and the initial exposure to a pCa 5.4 solution was made to obtain an initial response, which was used as a reference for determining the stability of the strand during the experiment (in these experiments this response changed by less than 10% for all but one strand in which it changed by 14%). Strands were exposed to solutions of pCa from 7.00 to 4.75, and the force at each pCa was recorded. The strand was then superfused with relaxing solution. The baseline at pCa 9.0 was determined several times during the
experiment. After a change in solution, force reached a steady-state level in 10–60 seconds.

Characterization of Troponin T Isoform Distribution in Each Strand

After the force–pCa data were obtained, the free length of the strand between the held ends was removed, placed in sample buffer, and stored at −80°C. Subsequently, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of 7.5% and 10% polyacrylamide gels was used to resolve the proteins by using a modified Laemmli protocol.11,13 The sources for electrophoresis reagents were as described previously.13 Silver staining of 10% polyacrylamide gels was performed as described by Schachat et al.26 Tropomyosin and cardiac troponin I were identified based on their electrophoretic mobilities, which had been determined from Western blots of ventricular myocardium that had been probed with monoclonal antibodies to tropomyosin and to cardiac troponin I. Gels were scanned densitometrically (Ultrascan XL, LKB Corp.), and the areas under the densitometric profiles of troponin I and tropomyosin were determined by integration. The relative amount of cardiac troponin I in each strand was normalized by calculating the troponin I/tropomyosin ratio from the integrated density profiles.

Western blots were performed on the proteins of each strand that were transblotted from 7.5% polyacrylamide gels onto polyvinylidene difluoride membranes,13 and the proportions of the troponin T isoforms were determined. A monoclonal antibody, MAb 13-11, which recognizes a determinant unique and specific to cardiac troponin T,27 was used as the primary antibody, and an alkaline phosphatase–labeled rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used as the secondary antibody. 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega Corp., Madison, Wis.) were used as the color development substrates. Western blots were scanned densitometrically, and the areas under the troponin T density waveforms were determined by integration.14 We used densitometric scans of Western blots rather than scans of silver-stained gels to quantitate the proportions of the troponin T isoforms because the electrophoretic mobilities of actin, tropinin T1 (TnT1), and tropinin T2 (TnT2) are very similar, making it difficult to resolve them on the gels.11,13

Statistical Analysis

The developed force at each pCa was normalized with respect to force developed at maximum activation. The data from each experiment were fitted with the Hill equation using nonlinear regression (Statgraphics, Statistical Graphics Corp.), and pCa0 and the Hill coefficient were determined. Regression analysis was used to test relations between pCa0 and the proportions of the troponin T isoforms and the normalized amount of cardiac troponin I within the strand.

Results

Twenty strands were isolated from 16 neonatal rabbits, of which 12 strands from nine rabbits satisfied our acceptance criteria and from which we obtained both force–pCa and protein data. Their data are listed in Table 1. Figure 1 illustrates proteins from two strands resolved by SDS-PAGE and demonstrates the presence of different relative amounts of the troponin T isoforms and similar proportions of cardiac troponin I to tropomyosin. Figure 2 illustrates the force–pCa relations of two strands studied at the same sarcomere length, the fit of these data with the Hill equation, and the corresponding densitometric scans of Western blots of their proteins probed with the cardiac-specific troponin T antibody MAb 13-11. These two strands characterize the span of the range of our results in both force–pCa relations and in relative amounts of the troponin T isoforms.

The relative amounts of the four troponin T isoforms (TnT1–TnT4), obtained from Western blots probed with MAb 13-11, varied among strands (see Table 1). The predominant isoforms were TnT2 and TnT4, with TnT1 and TnT3 making up on the average about 13% of the total troponin T. The relation between the percentage of total troponin T com-
posed of the isoform TnT₂ and pCa₅₀ for all strands is illustrated in Figure 3. The pCa₅₀ versus percent TnT₂ relation was positive and significant (p=0.037). Our data did not show a relation between the Hill coefficient and pCa₅₀ or percent TnT₂.

Changes in the cardiac troponin I/troponin ratio were used to search for possible differences among strands in relative amounts of slow skeletal troponin I and cardiac troponin I. The amounts of cardiac troponin I and troponin, based on their densitometric analysis, were well correlated (r=0.98, p<0.001), and the ratio of cardiac troponin I to tropomyosin, obtained from densitometric scans of silver-stained gels, was 0.96±0.24 (mean±SD). This ratio did not reveal evidence that differences in the expression of skeletal and of cardiac troponin I among our strands affected the force–pCa data. Neither pCa₅₀ nor the Hill coefficient correlated with the cardiac troponin I/troponin ratio, as would be expected if the strand-to-strand variability in the force–pCa relations had been due to changes in relative amounts of cardiac and skeletal troponin I.

**Discussion**

The relation between the sensitivity of cardiac myofilaments to calcium and troponin T isoform expression is an important new observation. Our results suggest that, in addition to its structural role in binding troponin I and C to troponym, troponin T has an important functional role in the modulation of cardiac contraction. This hypothesis is consistent with the findings of Schachat et al.⁹ in fast skeletal muscle that troponin T isoforms affect the force–pCa relation, Tobacman and Lee⁸ that cardiac troponin T isoforms have a small but significant effect on myofibrillar ATPase activity, and Anderson et al.⁷ that in ventricular myocardium from diseased and normal human hearts a relation exists between disease-associated changes in troponin T expression and myofibrillar ATPase activity.

Our results show that myofilament sensitivity to calcium increases with the relative amount of TnT₂, or alternatively, sensitivity decreases with an increase in TnT₄. Since TnT₂ decreases and TnT₄ increases with development,¹¹,¹³ these findings imply that a
developmental decrease in the sensitivity of rabbit cardiac myofilaments to calcium occurs. Such a change is consistent with the observations of Ventura-Clapier et al.,18 who found that pCa\(_{50}\) in rabbit left ventricular myofibrils fell rapidly from late fetal to early neonatal life (although pCa\(_{50}\) subsequently increased with further development) and those of McAuliffe et al.,19 who found that the sensitivity of rabbit myocardium to calcium decreased during postnatal life.

In the rat a developmental decrease in the pCa\(_{50}\) of single isolated fetal and adult myocytes has been found,20 and a developmental switch in expression of the two rat cardiac troponin T isoforms has been described during this period.10,15 In a recent abstract Reiser et al.17 found rat ventricular trabeculae and Purkinje strands from neonatal hearts to be more sensitive to calcium than those of adult heart, which correlated with a change in troponin T from the neonatal to the adult form.

In our present study, although myocardial preparations were obtained from animals in a very narrow postnatal period of development, fairly large differences were observed among strands in the proportions of their troponin T isoforms. These differences suggest the possibility that strand-to-strand variations in isoforms of other contractile proteins may also be present. This possibility is more likely to be a factor in the interpretation of results from preparations that span broader ranges of development, for example, in the studies of McAuliffe et al.,16 Ventura-Clapier et al.,18 and Reiser et al.17 Maturational changes in troponin C do not seem likely to be a significant contributor to differences in myofilament function: troponin C is expressed as a single form throughout development in chicken and mammalian heart.19,29 Although a switch in myosin heavy chain expression occurs in postnatal life in rabbit heart, little change appears to take place over the course of the few postnatal days from which our preparations were obtained30; more importantly, myofilament composition of myosin heavy chains has been shown not to affect the force–pCa relations of rabbit myocardium.31 In the mammalian heart, α- and β-tropomyosin are expressed32; however, little change in the relative amounts of the two isoforms has been described during late fetal and early neonatal life32 with the predominant form being the α-tropomyosin. In our preparations, tropomyosin was resolved on gels as one predominant peak whose electrophoretic mobility did not vary among the strands. Schachat et al.19 suggested that differences in the relative amounts of α- and β-tropomyosin did not appear to be major factors affecting the force–pCa relation in isolated fast skeletal muscle fibers.

Posttranslational changes in cardiac troponin I could have contributed to differences in pCa\(_{50}\) among strands. The cAMP-dependent phosphorylation of cardiac troponin I has been shown to affect the sensitivity of cardiac myofilaments to calcium.5,33 It is through this mechanism that β-adrenoceptor agonist stimulation causes a decrease in the sensitivity of the myofilaments to calcium. We were unable to use two-dimensional gel electrophoresis to determine the presence of phosphorylation because of the small size of the preparations.

The use of strands with small cross sections was dictated by our desire to avoid gradients in energy supply across the strand diameter during measurement of the force–pCa relation. Furthermore, we used only part of each strand (the free length between the anchored ends) in protein determinations to avoid contamination with proteins from the adjacent ventricular wall and to ensure that the protein and force–pCa data were obtained from the same preparation. This approach was dictated by our previous findings that the proportions of the cardiac troponin T isoforms varied from one region of the right atrium to another14 and that differences exist in the same heart between the proportions of the isoforms from the ventricular wall and from ventricular strands (unpublished observation).

Previous studies have suggested that circulating catecholamines are high at birth and then fall during the first few days after birth.34 This time course would suggest that a decrease in the sensitivity of cardiac myofilaments to calcium that results from cAMP-dependent phosphorylation of troponin I would be more prominent at birth. In considering these potential effects on myofilament sensitivity, we recall that TnT\(_2\) decreases during postnatal life11,13,16 so that myocardium with a relatively high content of this isoform is at an earlier stage of this programmed change in the troponin T isoforms. Consequently, it would be expected that strands with a higher amount of TnT\(_2\) are more likely to contain phosphorylated troponin I, which should result in decreased, not increased, sensitivity of the myofilaments to calcium. Yet we found that myofilaments with a higher proportion of TnT\(_2\) are more sensitive to calcium. Although these arguments support the idea that troponin I phosphorylation did not contribute to our results but rather may have obscured a greater sensitivity to calcium than what we observed, other studies that focus on posttranslational modifications of troponin I need to be performed to confirm these arguments.

Troponin I expression has been found to change with maturation in rat heart.35,36 Specifically, slow skeletal muscle troponin I is expressed contemporaneously with cardiac troponin I in immature rat heart.36 Consequently, changes in the relative expression of cardiac and slow skeletal troponin I could affect the sensitivity of the myofilaments to calcium. Although functional differences between slow skeletal and cardiac troponin I are not known, the slow skeletal form lacks the cAMP-dependent phosphorylation site.36 We assessed differences in the relative amounts of slow skeletal and cardiac isoforms among the strands by comparing cardiac troponin I to tropomyosin content. The two isoforms of troponin I have sufficient differences in their electrophoretic mobilities36 that identification and quantitation of the cardiac isoform on 10% polyacrylamide gels will not be contaminated by comigrating slow skeletal muscle isoform. The constancy of the troponin I/tropomyo-
sin ratio and the fact that it was not related to the 
$pC_{a_0}$ indicated to us that differences among the 
strand force-pCa relations are unlikely to be due to 
switching in troponin I expression from a slow skele-
tal muscle form to a cardiac form.

Thus, our findings indicate that a relation exists 
between the force-pCa characteristics of a strand 
and the troponin T isoforms it expresses, suggesting a 
functional role for troponin T in modulating the 
sensitivity of myofilaments to calcium. Whether these 
effects depend on troponin T binding to troponin I, 
troponin C, tropomyosin, or an interaction among 
them all remains to be investigated.

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Key Words • thin filament regulatory proteins • myofilaments 
• heart • calcium
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