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Development and Cardiac Contractility: Cardiac Troponin T Isoforms and Cytosolic Calcium in Rabbit

SHANNON J. MCCALL, RASHID NASSAR, NADIA N. MALOUF, ALEISTER J. SAUNDERS, ANNETTE E. OAKELEY, PAUL M. HENDERSON, R. JOHN SOLARO, GARY J. PIELAK, KENNETH A. ALEXANDER, AND PAGE A.W. ANDERSON

Departments of Pathology [S.J.M.] and Pediatrics [R.N., A.E.O., PAW.A.], Duke University Medical Center, Durham, NC 27710; Departments of Pathology [N.N.M] and Chemistry [G.J.P.], University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; Department of Bioscience and Biotechnology [A.J.S.], Drexel University, Philadelphia, PA 19104; Capital Family Medicine [P.M.H.], Wakefield Medical Care, Raleigh, NC, 27609; Department of Physiology and Biophysics [R.J.S.], University of Illinois at Chicago, Chicago, IL 60612; Department of Pediatrics [K.A.A.], University of Chicago Hospitals, Chicago, IL 60637

ABSTRACT: Cardiac contractility depends on calcium sensitivity of the myofilaments and cytosolic free calcium concentration ($[Ca^{2+}]_i$) during activation. During development, the cardiac troponin T isoform $cTnT_1$ is replaced by shorter cTnT isoforms, including $cTnT_4$, and changes occur in other myofibrillar proteins and in calcium regulation. We expressed rabbit recombinant (r)$cTnT_1$ and $rcTnT_4$ in *Spodoptera frugiperda* cells and determined their effect on calcium binding to TnC in solution and on the calcium sensitivity of myofilaments in skinned rabbit ventricular fibers *in vitro*. We measured $[Ca^{2+}]_i$ and L-type calcium current (I_{Ca}) in ventricular myocytes from 3-wk-old and adult rabbits. The dissociation constant (K_d) of Ca-Tn_{cTnT1} in solution was smaller than that of Ca-Tn_{cTnT4} (mean ± SE: 0.52 ± 0.08 μmol/L *versus* 0.83 ± 0.09 μmol/L). The Ca^{2+} sensitivity of force development was greater in fibers reconstituted with $rcTnT_1$ (pCa_{50} 6.07 ± 0.04) than those reconstituted with $rcTnT_4$ (pCa_{50} 5.75 ± 0.07). Systolic $[Ca]_i$ was lower in 3-wk-old than adult cells (443 ± 35 nmol/L *versus* 882 ± 88 nmol/L) as was I_{Ca} (5.8 ± 0.9 pA/pF *versus* 14.2 ± 1.6 pA/pF). The higher calcium sensitivity of Tn-Ca binding and of force development conferred by $rcTnT_1$ suggest that higher neonatal $cTnT_1$ expression may partially compensate for the lower systolic $[Ca^{2+}]_i$. (***Pediatr Res* 60: 276–281, 2006**)

Myocardial contractility increases with development. Adult myocardium develops more force than fetal myocardium (1). Adult cardiac myocytes shorten further and faster than immature myocytes (2). Potential contributors to these differences include developmental increases in calcium ($[Ca^{2+}]_i$) transients and calcium sensitivity of myofilaments (1,3). Calcium transients increase postnatally in the rabbit (4). The calcium sensitivity of myofilaments, however, decreases with development in the rat, chick, and rabbit (5–7); this fall could result from a developmental switch in isoform expression of cTnT, a thin filament protein essential for Ca^{2+}-regulated force development (8). Expression of $cTnT_1$ and $cTnT_2$ falls and that of $cTnT_3$ and $cTnT_4$ increases with development in the rabbit.

We expressed recombinant rabbit (r)$cTnT_1$ and $rcTnT_4$ in Sf9 cells and compared their effects on Ca^{2+}-binding of isolated troponin in solution and in skinned muscle fibers *in vitro*. Further, we compared calcium transients and L-type calcium currents (I_{Ca}) in cardiac myocytes from 3-wk-old rabbits expressing $cTnT_1$ and from adults that express predominantly $cTnT_4$ and no $cTnT_1$.

We found that (a) in solution, Tn comprising $cTnT_1$ (Tn_{cTnT1}) has higher calcium affinity than Tn comprising $cTnT_4$ (Tn_{cTnT4}), (b) fibers reconstituted with $cTnT_1$ were more sensitive to calcium than those reconstituted with $cTnT_4$, and (c) peak $[Ca^{2+}]_i$ and I_{Ca} were significantly greater in adult than in 3-wk-old myocytes. Our data provide no evidence that incorporation of Tn into the myofilaments alters Tn-calcium binding affinity.

By imparting to the myofilaments a higher calcium sensitivity, the higher expression of $cTnT_1$ and $cTnT_2$ in immature myocytes would compensate partially for their smaller $[Ca^{2+}]_i$ transients.

MATERIALS AND METHODS

Animals used in this study were handled according to the animal welfare regulations of Duke University (Durham, NC). The protocol was approved by the Institutional Animal Care and Use Committee. The rabbits were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine i.p.), and the heart was removed to obtain ventricular muscle fibers or isolated myocytes.

Preparation of cTnT. cTnT isoforms were expressed in Sf9 cells using the baculovirus system (Invitrogen). The cDNA of rabbit $cTnT_1$ (9) was cloned at the *EcoR* I restriction site into pVL1392 by releasing the cDNA from pBSII KS(+) with *Hind*III and *Kpn*I and cloning it into pSP72 (Promega) where a second *Xba*I site was added. The $cTnT_1$ cDNA was then excised with *Xba*I and cloned into pVL1392. Orientation of inserts and sequences encoding $cTnT_1$ and $cTnT_4$ were verified by polymerase chain reaction and DNA sequencing. SF9 cells were transfected with pVL1392-containing $cTnT_1$ cDNA or pVL1393-containing $cTnT_4$ cDNA and infected with wild-type

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Correspondence: Page A.W. Anderson, M.D., Department of Pediatrics, P.O. Box 3218, Duke University Medical Center, Durham, NC 27710; e-mail: ander005@mc.duke.edu
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Abbreviations: IAANS, 2-[4′-(iodoacetamido)aniline]-naphthalene-6-sulfonate; **MOPS,** 3-(*N*-morpholino)propane-sulfonate

Ac*M*PEV (Invitrogen). Clones were identified using monoclonal antibody (MAb) 13-11, a cTnT-specific MAb, and Western blots (10).

Sf9 cells (3–4 $\times 10^6$ cells/mL) were infected with $cTnT_1$ or $cTnT_4$ recombinant baculovirus. cTnT was purified according to Potter (11), and frozen at −70°C in 6 mol/L urea.

Circular dichroism. CD spectra for $cTnT_1$ and $cTnT_4$ were obtained at 25°C (0.5-nm steps with 1-nm bandwidth) in a buffer containing 0.5 mol/L KCl and 10 mmol/L potassium phosphate, and 3 mg/mL protein, pH 7.2.

Preparation of cTnC and cTnI. Human cTnC was expressed in BL21 (DE3) *Escherichia coli* using an expression vector provided by Merck and Co. Inc. and purified (12). Recombinant mouse cTnI was expressed using a pET-3d vector and purified using established methods (13). Stock protein solution concentrations were measured using quantitative amino acid analysis.

2-[4′-(Iodoacetamido)aniline]-naphthalene-6-sulfonate (IAANS) labeling of cTnC. Purified cTnC was dissolved in buffer A (0.2 mol/L KCl, 30 mmol/L 3-(*N*-morpholino)propane-sulfonate (MOPS), pH 7.0) to a final concentration of 50 μmol/L, and dialyzed serially against buffer A containing 1 mmol/L DTT and buffer A containing 6 mol/L urea without DTT (1,4-Dithiothreitol). cTnC was labeled using a 15-fold molar excess IAANS (Molecular Probes, Eugene, OR). The labeling stoichiometry was determined by 326-nm UV absorbance (IAANS molar extinction coefficient 26,000). Absorbance of cTnC at 280 nm was corrected for fluorophore absorbance (14). Labeling reactions yielded 2 mol bound IAANS/mol cTnC.

Preparation of soluble troponin complexes. Binary and ternary troponin complexes were formed at 20°C in titration buffer (in mmol/L: KCl 400, ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′ (EGTA) 1, MOPS 25, DTT 1.5, pH 7.2). Molar ratios of IAANS-cTnC:cTnI:cTnT were 1:2:2 to avoid dimer formation. Complexes of IAANS-cTnC:cTnI, IAANS-cTnC:$cTnT_1$, and IAANS-cTnC:$cTnT_4$ were prepared similarly.

Calculation of $[Ca^{2+}]_{free}$. $[Ca^{2+}]_{free}$ was calculated using CATCONC (15) software and the logarithmic association constants: H^+:$EGTA^{4-}$ 9.47, H^+:$HEGTA^{3-}$ 8.85, H^+:H_2EGTA^{2-} 2.66, H^+:H_3EGTA^- 2.0; Ca^{2+}:$EGTA^{4-}$ 10.97, Ca^{2+}:$HEGTA^{3-}$ 5.29, Mg^{2+}:$EGTA^{4-}$ 5.21, Mg^{2+}:$HEGTA^{3-}$ 3.43.

Ca^{2+}-troponin affinity in solution. Fluorescence measurements were made using a SLM 8100 spectrofluorometer (SLM/Aminco). Titration buffer (mmol/L): KCl 0.4, EGTA 1, and MOPS 25, pH 7.2, 20°C. Fluorescence emission spectra were obtained using 335-nm excitation (excitation and emission bandwidths: 4 and 32 nm, respectively). Uncorrected fluorescence intensities between 400 and 500 nm were integrated and the fraction of Ca^{2+}-bound IAANS-cTnC (F_b) calculated:

$$F_b = (I_{obsv} - I_{EGTA})/(I_{sat} - I_{EGTA}) \quad (1)$$

where I_{obsv} is the integrated fluorescence intensity observed after each addition of Ca^{2+}, I_{EGTA} is the integrated fluorescence in the absence of Ca^{2+}, and I_{sat} is the fluorescence under saturating Ca^{2+}). Data were fit to a form of the Hill equation:

$$F_b/F_{b\ max} = Ca_{nH}/(Ca_H + K_d) \quad (2)$$

where n_H is the Hill coefficient and K_d is the dissociation constant).

Force-pCa relationship. Right ventricular muscle fibers from adult rabbit hearts (16) were skinned (0.5% Triton X-100) in relaxing solution. Compositions of relaxing (pCa 9) and pCa 4.5 solutions (6) and their preparation were as described previously (17), except pMg was 3.0, and all solutions contained 0.1 mg/L pepstatin A. Apparent Ca-EGTA stability constant used was 3.702×10^6 L/mol.

Fibers were mounted on a force transducer (Güth OP1M (18)) at 22.0°C and sarcomere length of 2.3 μm (determined by light diffraction). Active force was determined *versus* pCa.

Dialysis of rcTnT. rcTnT was dialyzed at 4°C for 24 h, first against 2 mol/L urea and 1 mol/L KCl, then sequentially against 1, 0.8, 0.6, and 0.4 mol/L KCl, all containing 20 mmol/L MOPS, 1 mmol/L DTT, and 0.1 mg/L pepstatin A, pH 7.0.

Solutions for skinned preparation experiments. The washing solution used (in mmol/L) was KCl 100, $MgCl_2$ 5, MOPS 20, DTT 0.5, pepstatin A 0.1, pH 7.0. The extraction solution was KCl 250, EGTA 5, $MgCl_2$ 5, MOPS 20, DTT 0.5, pepstatin A 0.1 (pH 6.5), and 1–3 mg/mL $rcTnT_1$ or $rcTnT_4$. The reconstitution solution was 2.5 mg/mL cTnI-cTnC complex added to either (in mmol/L) KCl 100, EGTA 5, $MgCl_2$ 5, DTT 0.5, pepstatin A 0.1 mg/L, MOPS 20, or imidazole 120, KCl 29, $MgCl_2$ 16, EGTA 7, ATP 5, phosphocreatine 16 (pH 7).

Extraction-reconstitution assay. We superfused the preparation in washing solution (10 min), then in extraction solution (70–90 min) to remove troponin (19). Loss of Ca^{2+}-regulated force was verified using pCa 4.75. The fiber was then washed (10 min) and placed in reconstitution solution 75 min). In some preparations, extraction-replacement was performed in one step: preparations were exposed for 100 min to extraction solution containing Tn_{cTnT1} or Tn_{cTnT4} complex. The force-pCa relationship was measured, the fibers placed in sample buffer, and their proteins resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. Western blots were probed with MAb 13-11 (10,20).

Cell isolation. Myocytes were isolated following the procedure of Mitra and Morad (21). The digestion solution for 3-wk-old animals contained 0.5% collagenase (Worthington type II) and 0.006% protease (Sigma Chemical Co., type XIV) in 50 mL of physiologic solution (no added calcium); for adult animals, we used 1% collagenase and 0.006% protease.

Electrophysiology and calcium measurement. Cells were studied under a Zeiss IM microscope using a Nikon 40x, 0.85-N.A. Fluor objective. Electrophysiologic data were acquired using Axopatch 200A, Digidata 1200, and pClamp 6.0 (Axon Instruments) (in early experiments, we used a Dagan Corporation Model 8900). FluoroPlex-III system (Tracor Northern) was used for fluorescence measurements (excitation wavelengths 350 and 380 nm, chopper rate, 60 s^{-1}). Ventricular myocytes were whole-cell clamped (22) at room temperature (pipette resistance 1.8–2.5 MΩ). I_{Ca} was acquired at 10 or 20 kHz (2-kHz, four-pole, low-pass Bessel filter). Capacitance and 70–80% series-resistance compensation were applied. Holding potential (V_H) was −80 mV; five conditioning 400-ms pulses to +10 mV at 4-s intervals standardized sarcoplasmic reticulum calcium load. V_H from the end of the last conditioning pulse to the 400-ms test pulse was −40 mV (to inactivate sodium and T-type calcium channels). Resting potential (E_m) was recorded immediately upon patch rupture and $[Ca^{2+}]_i$ and I_{Ca} 5–10 min post-rupture.

Solutions for cardiac myocyte experiments. The extracellular solution (in mmol/L) was NaCl 141.8, KCl 5.1, $MgCl_2$ 1.0, dextrose 12, MOPS (acid) 3.75, MOPS (Na salt) 6.25, and $CaCl_2$ 2.0, pH 7.4 and the pipette solution was KCl 143, Na_2ATP 5.1, $MgCl_2$ 5.04, MOPS (acid) 10, EGTA 0.002, and K_5fura-2 0.05 or 0.1, pH 7.2.

Calibration of fura-2 fluorescence. We carried out in-cell calibration at room temperature as described by Li *et al.* (23).

Data analysis. $[Ca^{2+}]_i$ was calculated according to Grynkiewics *et al.* (24) using Ca-fura-2 K_d of 200 μmol/L. Background was subtracted before calculating fluorescence ratio. I_{Ca} was measured as negative peak minus steady-state current at 300 ms; this was equal, both in adult and 3-wk-old animals, to the (200 μmol/L) Cd^{2+}-sensitive current. Cell capacitance was calculated from the time constant of response to small (±5 mV) test pulses from $V_h = -80$ mV sampled at 250 kHz (10-kHz filter) before compensation.

We used the notation $pCa_{50} = -\log_{10}[Ca^{2+}]$ for the pCa that elicited half-maximal tension, and $pK_d = -\log_{10}K_d$ for protein solutions.

Statistics. Means or fitted values ± their standard error (SE) are given unless otherwise stated. We used an unpaired *t* test or *t* test for unequal variance. The paired *t* test was used to assess the difference in binding of Ca^{2+} to IAANS-Tn_{cTnT1} and IAANS-Tn_{cTnT4}. I_{Ca} was corrected for cell capacitance by linear regression; I_{Ca} density was based on C_m of 80 pF. Statistical analysis was carried out using R (http://www.R-project.org)

RESULTS

Characterization of recombinant protein. The purity and integrity of $rcTnT_1$ and $rcTnT_4$ were verified by comparison to native cTnT using SDS-PAGE and Western blots (Fig. 1*A*), and CD spectra, which exhibited two minima at 208 and 222 nm and were like the spectrum of native cTnT (25).

Ca^{2+} concentration effects on fluorescence of IAANS-cTnC, IAANS-cTnC:cTnI, IAANS-cTnC:cTnT, and IAANS-cTnC:cTnI:cTnT containing $rcTnT_1$ or $rcTnT_4$. We examined the effects of binding of Mg^{2+} or Ca^{2+} to cTnC high-affinity sites III and IV and of Ca^{2+} to the low-affinity site II on IAANS-cTnC fluorescence. In the absence of Mg^{2+}, IAANS-cTnC fluorescence intensity decreased slightly between pCa 8.5 and 6.0, then increased as pCa was further reduced to 3.2, the increase being attributable to Ca^{2+} binding to site II (Fig. 2*A*). The apparent K_d of free IAANS-cTnC:Ca^{2+} was 0.104 μmol/L for the high-affinity sites and 9.7 μmol/L for the low-affinity site (data not shown).

The IAANS-cTnC:cTnI complex was titrated with Ca^{2+} in the presence of 4 mmol/L Mg^{2+} to saturate both cTnC high-affinity sites III and IV (Fig. 2*B*). Like Putkey *et al.* (26), we found a minimal decrease in fluorescence with increasing

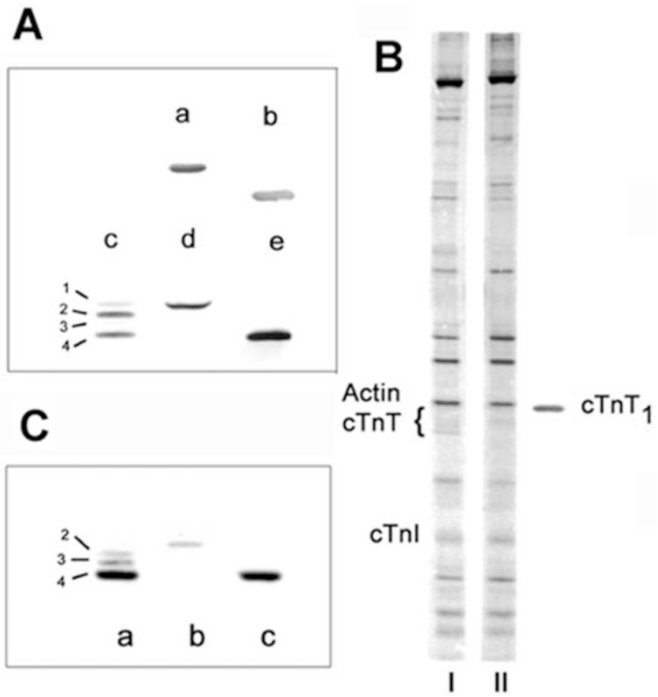

Figure 1. Recombinant and native cardiac troponin T. *A*, Silver stains comparing electrophoretic mobilities of purified recombinant (r)$cTnT_1$ (*a*) and $rcTnT_4$ (*b*). Western blots, probed with MAb 13-11, which recognizes an epitope specific to cTnT, compare electrophoretic mobilities of purified $rcTnT_1$ (*d*) and $rcTnT_4$ (*e*) to the four native isoforms ($cTnT_{1-4}$) from 2-d-old rabbit myocardium (*c*). cTnT isoform expression in neonatal heart (*c*) differs from that of adult rabbit heart (*C*) in which $cTnT_1$ is absent and TnT_4 is the major isoform. *B*, Proteins from a control (I) and an experimental (II) fiber (8.5% acrylamide gel). Endogenous cTnT isoforms in II were replaced with $rcTnT_1$. The third lane contains $rcTnT_1$. *C*, Western blots (probed with MAb 13-11) of a control adult fiber (*a*) and two experimental fibers (*b*, *c*), reconstituted, with $rcTnT_1$ (*b*) and $rcTnT_4$ (*c*). In the experimental fibers, only $rcTnT_1$ (*b*) and $rcTnT_4$ (*c*) are evident.

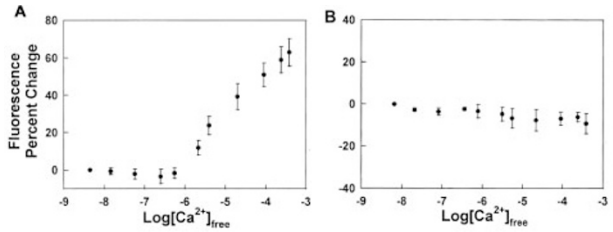

Figure 2. Effect of Ca^{2+} on fluorescence of IAANS-labeled cTnC (*A*) and IAANS-cTnC:cTnI complex (*B*) expressed as percentage of change relative to initial fluorescence. Data shown as mean ± standard deviation (five titrations).

$[Ca^{2+}]$. Fluorescence of IAANS-cTnC:$cTnT_1$ and IAANS-cTnC:$cTnT_4$ complexes did not change with $[Ca^{2+}]$ (data not shown).

The fluorescence intensity of IAANS-Tn_{cTnT1} and IAANS-Tn_{cTnT4} decreased with $[Ca^{2+}]$: K_d for IAANS-Tn_{cTnT1}:Ca^{2+} was 0.52 ± 0.08 μmol/L and for IAANS-Tn_{cTnT4}:Ca^{2+} 0.83 ± 0.09 μmol/L ($p = 0.001$; paired *t* test, five determinations); n_H were 1.0 ± 0.1 and 1.3 ± 0.2, respectively (Fig. 3).

Extraction-reconstitution experiments. Mean pCa_{50} of the fibers with endogenous cTnT was 5.99 ± 0.07 and n_H was 2.3 ± 0.5 ($n = 10$). SDS-PAGE (Fig. 1*B*) and Western blots (Fig. 1 *C*) demonstrated replacement of endogenous cTnT by

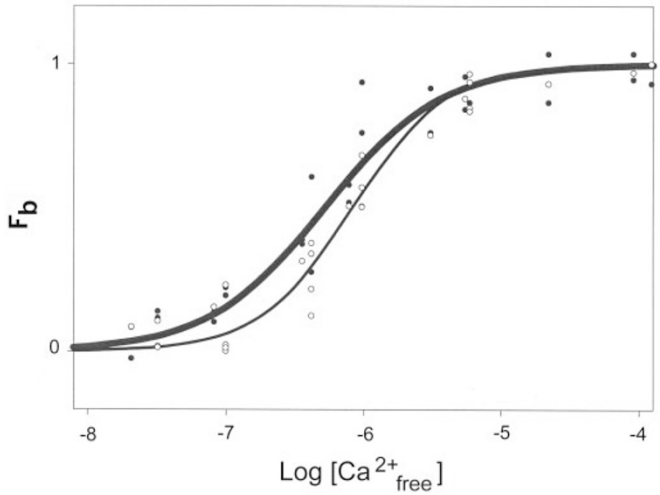

Figure 3. Calcium binding to TnC. Fraction of Ca^{2+}-bound IAANS-cTnC (F_b) as a function of $\log_{10}[Ca^{2+}]$ for Tn_{cTnT1} (*thick line*, *filled symbols*), and Tn_{cTnT4} (*thin line*, *open symbols*) illustrates the greater calcium affinity of Tn_{cTnT1}.

$rcTnT_1$ or $rcTnT_4$. pCa_{50} postreconstitution was 6.07 ± 0.04 ($n = 6$) for $rcTnT_1$, and 5.75 ± 0.07 ($n = 4$) for $rcTnT_4$, $p = 0.003$ (Fig. 4) and n_H was 2.4 ± 0.4 and 1.9 ± 0.1, respectively ($p = 0.34$).

Comparison of Tn-Ca binding in solution and in myofilaments. To assess whether incorporation of troponin into the myofilaments changed troponin:calcium affinity, we compared the differences (Δ) in Gibbs free energy (ΔG): $\Delta\Delta G_{solution} = \Delta G_{TnT1} - \Delta G_{TnT4} = 2.303RT\cdot(pK_{d\ [TnT1]} - pK_{d\ [TnT4]})$ for Tn in solution, and $\Delta\Delta G_{muscle} = 2.303RT\cdot(pCa_{50[TnT1]} - pCa_{50[TnT4]})$ in the myofilaments (R, universal gas constant; T, absolute temperature). This assumes that at pCa_{50}, calcium binding sites of myofilament-bound cTnC are half-occupied. The two quantities were not significantly different: $\Delta\Delta G_{solution}$

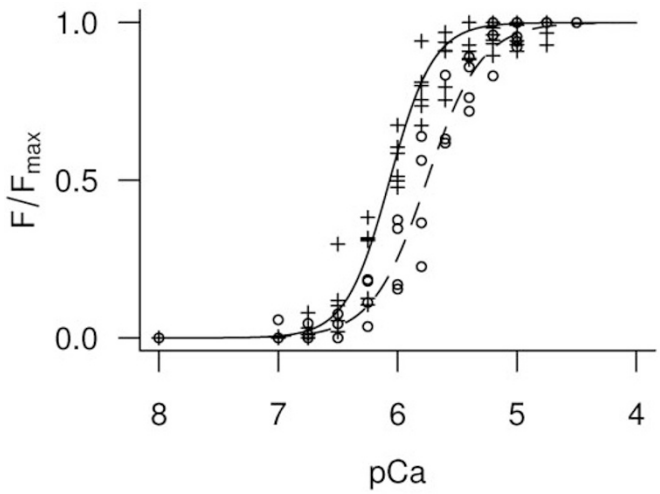

Figure 4. Force *vs* pCa. Normalized force *vs* pCa data for fibers in which the endogenous cTnT was replaced with either $rcTnT_1$ (+, *solid curve*) or $rcTnT_4$ (○, *dashed curve*) illustrates the greater calcium sensitivity of force development in fibers containing $rcTnT_1$. The curves correspond to mean values for pCa_{50} and n_H.

was 0.29 ± 0.07 (95% confidence interval: 0.13–0.46) kcal/mol, and $\Delta\Delta G_{muscle}$ was 0.42 ± 0.10 (0.18–0.66) kcal/mol (Fig. 5).

Ventricular myocytes. Body weights of 11 adult and 16 3-wk-old rabbits from which myocytes were obtained were 2840 (standard deviation [SD] = 300) g and 300 (SD = 100) g. Cells from 13 animals yielded $[Ca]_i$ and electrophysiologic data; cells from 13 animals yielded $[Ca]_i$ data.

Peak $[Ca^{2+}]_i$ in adult myocytes was twice that in 3-wk-old myocytes (Table 1, Fig. 6*A*). Diastolic $[Ca^{2+}]_i$ and resting potentials in adult and 3-wk-old myocytes were not significantly different. Membrane capacitance (C_m) was significantly greater in adult myocytes due to their larger cell size (2). I_{Ca} and I_{Ca} density (Table 1, Fig. 6*B*, *C*) were both greater in adult myocytes. The peak of the I_{Ca} *versus* voltage curve in both age groups occurred at +10 mV (Fig. 6*C*).

Table 1. *Comparison of $[Ca]_i$, resting potential (E_m), C_m, and I_{Ca} between ventricular myocytes from adult and 3-wk-old rabbits*

	Adult	Immature
$[Ca]_{i\text{-}peak}$ (nmol/L)	882 ± 88 (10)	443 ± 35 (22)*
$[Ca]_{i\text{-}rest}$ (nmol/L)	134 ± 18 (10)	141 ± 9 (22)†
E_m (mV)	−78.1 ± 1.4 (9)	−75.4 ± 1.4 (19)†
C_m (pF)	147 ± 18 (7)	44 ± 3 (16)*
I_{Ca} (nA)	1.48 ± 0.15 (7)	0.27 ± 0.03 (16)*
I_{Ca} density (pA/pF)	14.2 ± 1.6 (7)	5.8 ± 0.9 (16)*

Significance of difference between adult and immature myocytes: * $p \leq 0.001$; † $p \geq 0.20$.

n is shown in parentheses.

DISCUSSION

Myocardial contractility has been shown to increase with development. The $[Ca^{2+}]_i$ transient increases postnatally, but the calcium sensitivity of the myofilaments decreases. cTnT isoform expression also changes with development and is altered by heart disease (9,27,28). Although these changes, with opposite effects on contractility, appear to be at cross-purposes, the greater calcium sensitivity of tension would seem to partially compensate for the lower $[Ca]_i$ in the immature myocyte.

Four cTnT isoforms, $cTnT_{1\text{-}4}$, expressed in the rabbit and human heart (9,27,29), are products of combinatorial alternative splicing of two exons (9). Expression of $cTnT_1$ peaks in fetal human (36% of total cTnT) (27) and neonatal rabbit heart. In adult rabbit heart, $cTnT_4$ is the dominantly expressed isoform, whereas in the normal adult human, only $cTnT_3$ is expressed (9,27). These changes in isoform expression suggest that cTnT isoforms have functional consequence.

cTnT isoforms in solution. We determined for the first time, in solution, that the affinity of Tn_{cTnT1} was greater than Tn_{cTnT4} and that this difference did not require incorporation of Tn into the myofilaments.

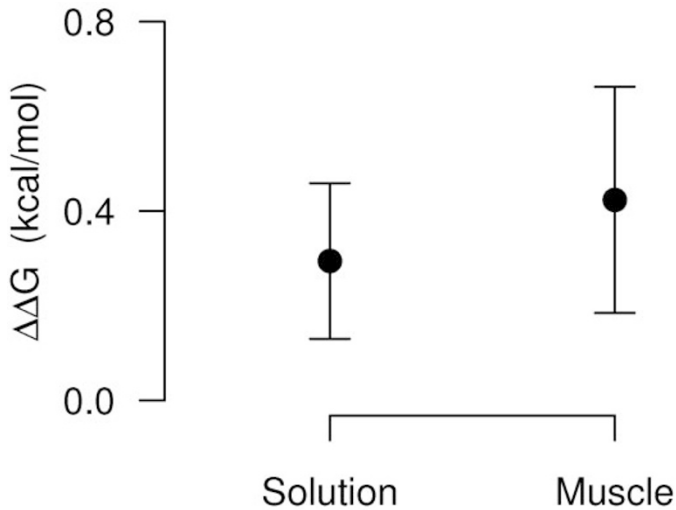

Figure 5. Difference in free energy (ΔΔG) of Ca^{2+} binding to cTnI:cTnC:$cTnT_1$ and to cTnI:cTnC:$cTnT_4$ in solution (Solution) and in fibers reconstituted with $rcTnT_1$ and $rcTnT_4$ (Muscle). Error bars: 95% confidence intervals.

Calcium titration did not affect IAANS-cTnC:cTnT fluorescence, although it affected IAANS:Tn fluorescence, the effect depending on the presence of $cTnT_1$ or $cTnT_4$, suggesting that cTnT isoform amino-terminal regions, which are distant from known cTnT, cTnC, and cTnI interaction sites, affect the interactions among cTnC, cTnI, and cTnT, and this effect requires cTnI.

cTnT isoforms in the myofilament: the force-pCa relationship. We found that the calcium sensitivity of active tension was higher in rabbit fibers reconstituted with rabbit $rcTnT_1$ than with rabbit $rcTnT_4$, in agreement with Gomes *et al.* (30), who examined the effects of human (h)cTnT isoforms on the force-pCa relationship of porcine fibers. There was an apparent rightward shift of the force-pCa relationship post-replacement. This may be due to our using mouse cTnI, which is 92% homologous with rabbit cTnI (we also used human cTnC, but that has 100% homology with rabbit cTnC). Also, extraction can affect other myofilament proteins, *e.g.* myosin binding protein C (31). Whatever the reason, both experimental groups would be affected similarly because the only difference between them was the cTnT isoform used. These results suggest that the developmental decrease in expression of $cTnT_1$ and $cTnT_2$ and increase in $cTnT_3$ and $cTnT_4$ contribute to the decrease in calcium sensitivity in rabbit and rat myocardium (7,32).

Developmental changes and species differences in isoform expression of slow skeletal (ss)TnI, cTnI, tropomyosin and myosin heavy chain (MHC) may contribute to differences in calcium sensitivity (3,33,34). The neonatal switch in expression from ssTnI to cTnI could lower calcium sensitivity (3,35,36) and modify cTnT isoform effects on tension (30). However, we found, in neonatal rabbit, a positive correlation between the sensitivity of myofilaments to calcium and $cTnT_2$ expression but no evidence of a difference in relative cTnI to ssTnI expression (16).

Comparison of calcium binding of CTnT isoforms in solution and the myofilaments. In the thin filament, the cTnT amino-terminus overlaps the head-to-tail binding of adjoining tropomyosin molecules, suggesting a structure-function relationship. We hypothesized that incorporation of troponin into the myofilaments would alter the calcium affinity induced by the troponin isoforms in solution. If so, ΔΔG between calcium binding to Tn_{cTnT1} and Tn_{cTnT4} in solution should differ from

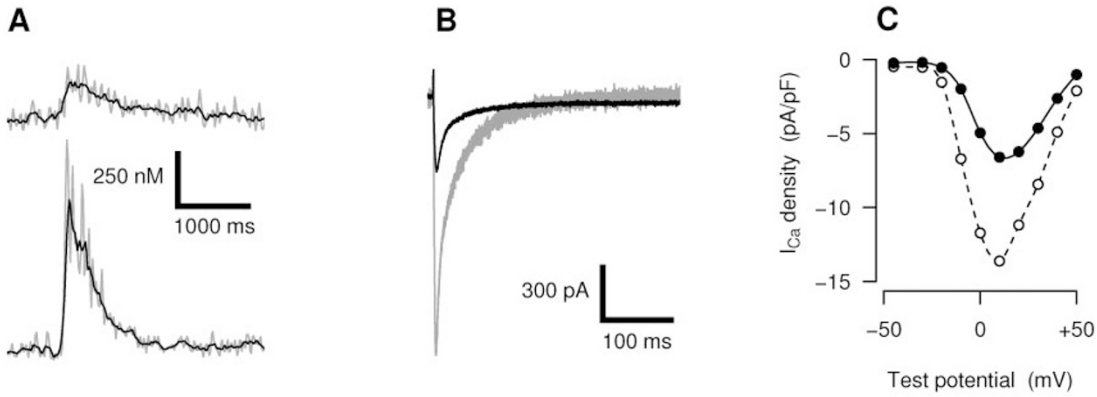

Figure 6. Comparison of calcium transients and L-type calcium currents from 3-wk-old and adult rabbit ventricular myocytes. *A*, Calcium transients from the 3-wk-old (*upper trace*) show much smaller transients than the adult. Diastolic levels were 155 and 103 nmol/L, respectively. Black traces: filtered. *B*, L-type calcium currents from the 3-wk-old (*black trace*) show a significantly smaller current than the adult (*gray trace*) myocyte. *C*, I_{Ca}-voltage relations from a 3-wk-old (capacitance 61 pF, *closed symbols*) and an adult (capacitance 109 pF, *open symbols*) myocyte.

that in myofilaments: we found, however, that the difference in $\Delta\Delta G$ between fibers containing $rcTnT_1$ and $rcTnT_4$ resulted mainly from the intrinsic differences in Tn_{cTnT1} and Tn_{cTnT4} and not from incorporation of troponin into myofilaments (Fig. 5).

Systolic $[Ca^{2+}]_i$, C_m, and I_{Ca} density were larger in adult than in 3-wk-old rabbit ventricular myocytes, in agreement with previous reports (37–39). In an earlier study (2), we found larger and faster sarcomere shortening in the adult myocyte, consistent with its higher systolic $[Ca^{2+}]_i$. We found no developmental difference in diastolic $[Ca^{2+}]_i$, consistent with our previous finding of no difference in resting sarcomere length (2).

SUMMARY

In solution and in myofilaments, the affinity of Tn_{cTnT1} for calcium was higher than that of Tn_{cTnT4}; our results provide no evidence that these affinities are modified upon incorporation of Tn into myofilaments. The higher calcium sensitivity of myofilaments from immature myocardium, due at least in part to developmental changes in cTnT isoform expression, may compensate for its smaller $[Ca^{2+}]_i$ transient: for the same $[Ca^{2+}]_i$, a small shift of the force-pCa curve can result in a dramatic difference in generated force, given the steepness of the curve in the activation region (Fig. 4).

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