Role of Cardiac Myosin Binding Protein C in Sustaining Left Ventricular Systolic Stiffening

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Abstract—Despite advances in the molecular biology of cardiac myosin binding protein-C (cMyBP-C), little is understood about its precise role in muscle contraction, particularly in the intact heart. We tested the hypothesis that cMyBP-C is central to the time course and magnitude of left ventricular systolic elastance (systolic stiffening), and assessed mechanisms for this influence in intact hearts, trabeculae, and skinned fibers from wild-type (+/+) and homozygous truncated cMyBP-C (t/t) male mice. cMyBP-C protein was not detected by gel electrophoresis or Western blot in t/t myocardium. cMyBP-C t/t ventricles displayed reduced peak elastance, but more strikingly a marked abbreviation of the systolic elastance time course, which peaked earlier (27.6±2.1 ms) than in +/- controls (47.8±1.6 ms). Control hearts reached only 42±4% of maximum elastance at the onset of ejection, with substantial further stiffening during ejection. This contrasted to t/t mutants, which reached 77±3% of peak elastance before ejection of peak. These unusual findings were not observed in alternative models involving severe cardiomyopathy, but were recapitulated in a cMyBP-C null mouse. The abbreviated elastance time course and lower peak were consistent with earlier time-to-peak trabecular tension, increased unloaded shortening velocity in t/t skinned muscle strips, and dramatically reduced myofilament stiffness at diastolic calcium concentrations. These results provide novel insights into the role of cMyBP-C in myocardial systolic mechanics. Abnormal sarcomere shortening velocity and abbreviated muscle stiffening may underlie development of cardiac dysfunction associated with deficient incorporation of cMyBP-C. (Circ Res. 2004;94:1249-1255.)

The contributions of cardiac myosin binding protein-C (cMyBP-C) to sarcomeric mechanical properties and force development have attracted considerable attention and speculation. cMyBP-C is known to reside in the thick filament at 43 nm repeats within the C-zone of the half sarcomere1–4 and to promote normal thick filament structure.5,6 The phosphorylation of cMyBP-C influences the arrangement of myosin heads7–9 and possibly modulates force production.10,11 It has been proposed that cMyBP-C trimerizes to form a collar around the thick filament2,3,7 with interdomain binding of C5 and C7 of one molecule to C8 and C10, respectively, of another.12 The structural role of cMyBP-C as a collar or as some other securing structure within the thick filament is attractive, because it suggests at least two possible functional roles: (1) the C-terminus, which binds to the myosin rod, to titin, and to other cMyBP-C, would mechanically stabilize the thick filament and facilitate force transmission between the crossbridges and the M-line, and (2) the N-terminus, which possesses phosphorylation sites and binding sites to myosin S2 and to actin, would modulate myosin interaction with actin.10–14 Despite the apparent significance of cMyBP-C and its phosphorylation to thick filament structure and function, thick filaments incorporating mutant cMyBP-C or even lacking cMyBP-C are capable of sustaining viable heart function in genetically engineered mice.15–20 Mouse models with marked deficiency or null for cMyBP-C progress to a dilated cardiomyopathy17,20 with reduced ejection and maximal LV end-systolic elastance despite preserved maximal rate of pressure rise.17 The latter measure is more typically reduced with heart failure. However, the effective absence of cMyBP-C reportedly enhances unloaded shortening velocity in skinned rat cardiomyocytes,21 increases contractile power and the rate of force redevelopment in skinned mouse myocardium.
and reduces longitudinal stiffness in skinned mouse myocardial strips under rigor conditions.\textsuperscript{23} Such data suggest that cMyBP-C might specifically affect sarcomeric mechanical properties such as stiffness and viscosity, thereby influencing systolic function. However, a mechanism linking these myocardial properties with the observed chamber dilation and cardiodepression has remained elusive.\textsuperscript{22}

In the present study, we elucidated the functional consequences of an effective lack of cMyBP-C on properties of myocardial mechanics in isolated muscle and intact chambers by studying homozygous (t/t) male mice whose mutant allele coded for a truncated cMyBP-C effectively absent from the sarcomere.\textsuperscript{17} We demonstrate an unusual and marked abbreviation of the time course of systolic stiffening (elasticity) in the LV of t/t mice compared with wild-type controls (+/+) and identical findings were obtained in cMyBP-C null mice (−/−).\textsuperscript{20,22} Further analyses of intact and skinned cMyBP-C t/t myocardium support a mechanism by which cMyBP-C critically contributes to calcium-dependent stiffening and internal loading on the sarcomere.

Materials and Methods

Mouse Models

All t/t and respective +/+ mice used in this study were male, aged 10 to 20 weeks, and of the 129-SvEv background.\textsuperscript{17} All −/− and respective +/+ mice were of either sex, aged 8 to 12 weeks, and of the 129Sv background.\textsuperscript{20} All experimental protocols were in accordance with Institutional Animal Care and Use Committees.

cMyBP-C Content

Protein expression of cMyBP-C in the t/t is reportedly low.\textsuperscript{17,23} We further tested this by 4% to 15% gradient SDS-PAGE electrophoresis and Western blotting (Figure 1). Whereas wild-type cMyBP-C protein was detected at ~150 kDa in the +/+ myocardium, the truncated cMyBP-C, which lacks the C8-C10 domains and should have migrated to ~130 kDa,\textsuperscript{17} was not detected. cMyBP-C was undetectable in t/t myocardium between ~40 to 200 kDa by Western blot or at any molecular weight based on Dot blot (Figure 1, inset) using two polyclonal antibodies known to detect the N-terminus of cMyBP-C.\textsuperscript{20,24} Thus, the amount of truncated cMyBP-C present in the t/t was below the sensitivity of detection, ie, <3% of that in +/+ as has been reported for this and other truncated cMyBP-C.\textsuperscript{17,23,24} There is no expressed cMyBP-C protein in −/− animals as reported.\textsuperscript{20}

We furthermore compared the relative densities of titin, myomesin, and M-protein between +/+ and t/t. Electrophoresis gels of 5% glyceraldehyde, 8% acrylamide, run at 75 and 200 V for 27 hours, and maintained at 4°C to 6°C were particularly sensitive to identifying proteins greater than 150 kDa. The relative optical densities of titin, myomesin, and M-protein normalized to MHC density after Coomassie stain and silver stain were not noticeably changed by the lack of cMyBP-C in the t/t (data not shown).

LV Function

LV function was characterized in situ using methods described previously.\textsuperscript{23} Mice were anesthetized with IP urethane (800 to 1000 mg/kg), etomidate (20 to 25 mg/kg), and morphine (1 to 2 mg/kg) and intubated. An external jugular vein was cannulated for IV fluids (12.5% human albumin). A 1.4F pressure-volume catheter was inserted retrograde along the LV long axis via the apex, and absolute volume was calibrated as described.\textsuperscript{26} End-systolic pressure-volume relations were recorded during transient obstruction of inferior vena cava inflow and used to determine the end-systolic elastance ($E_s$) and volume intercept ($V_i$) of the end-systolic pressure-volume relationship.\textsuperscript{26} The normalized time-varying chamber elastance was equal to $P(t)/V(t) - V_i/E_s$.

Intact Trabeculae

Ultrathin, uniform, nonbranched RV trabeculae were dissected leaving a block of tissue at one end from the right ventricular free wall and a small part of the valve at the other end to facilitate mounting on an isometric force-transducer apparatus as described.\textsuperscript{27} Average muscle dimensions were 86±8 μm wide, 66±6 μm thick, and 1.38±0.11 mm long. To avoid core hypoxia and the accumulation of waste products,\textsuperscript{27,28} no muscles greater than 100 μm in thickness or width were used. Cross-sectional area was calculated assuming an ellipsoid shape. Muscles were mounted and experiments performed at 37°C in Krebs-Henseleit solution as described.\textsuperscript{27}

Skinned Strips

LV skinned myocardial strips were studied as previously described.\textsuperscript{29} Papillary muscles were dissected to yield at least two thin strips (~140 μm diameter, ~800 μm length) with longitudinally oriented parallel fibers, skinned for 18 hours at 4°C, and stored at −20°C for no more than 4 days. Aluminum T-clips were attached to the ends of a strip ~300 μm apart. The strip was mounted between a piezoelectric motor and a strain gauge, lowered into a 30 μL droplet of relaxing solution maintained at 27°C, and incrementally stretched to and maintained at 2.2 μm sarcomere length.\textsuperscript{29} Strips were calcium activated from pCa 8.0 to pCa 4.5. Sinusoidal perturbations of amplitude 0.125% strip length were applied at 0.125 to 100 Hz.\textsuperscript{29}

Analysis

Variables sensitive to varying calcium were fit to the Hill equation using a nonlinear least squares algorithm (Sigma Plot 8.0, SPSS). All data are presented as mean±SEM, and significance by Student’s $t$ test reported at the $P<0.05$ and $P<0.01$ levels.

Results

Characteristics of cMyBP-C Mutant (t/t and −/−) Hearts

Both t/t and −/− mouse hearts had higher mass, lower in vivo ejection fraction (Table), and greater LV chamber end-diastolic dimension consistent with dilated cardiomyopathy.\textsuperscript{17,25} Maximal rate of pressure rise (dP/dt max) in the t/t and −/− was similar to that in respective +/+ controls, whereas
rate of pressure decline (−dP/dt_{max}) in t/t and −/− was markedly diminished (Table). 17,20 Resting hearts rates were similar in all groups (≈600 bpm), and the V3/V1 myosin ratio was significantly increased in the t/t and −/− mice compared with that for +/+ as reported previously. 17,20

**Influence of cMYBP-C on Time Course of LV Systolic Elastance**

Figure 2A displays example pressure-volume loops and relations for both genotypes. LV chambers were dilated in t/t with reduced stroke volume (loop width) and end-systolic elastance (Figure 2B). Figure 2C displays a normalized time course of ventricular elastance [E_{n}(t)] for both a +/+ and t/t heart, with time at onset-of-ejection (t_{oe}) and end-systole (t_{es}) denoted. In +/+, E_{n}(t) reached 42±3% of its peak value at t_{oe}, then continued to rise steadily to maximum at t_{es}. Hearts from t/t mice displayed a similar initial E_{n}(t) rise, but this peaked almost immediately after t_{oe} and then declined rapidly. The ratio of E_{n}/E_{max} (percent total chamber stiffening occurring before ejection) was therefore much higher in the t/t (Figure 2D), and duration of the ejection phase denoted as t_{es} was markedly abbreviated (27.6±2.1 versus 47.8±1.6 ms in +/+, P<0.001) in t/t hearts (Figure 2F). Results for the cMyBP-C null animals (−/−) were virtually identical, (t_{es}=28.2±2.2 versus 43.0±0.5 ms in littermate +/+ controls; P<0.001).

Figure 2E contrasts the E_{n}(t) kinetics in the −/− mouse heart to those from several murine models that each display substantial systolic dysfunction: autoimmune myocarditis, 30 LV-targeted MKK3a (p38) overexpression, 31 troponin I truncation mutation, 32 and a double desmin knockout. 33 Importantly, in addition to primary defects for each model, these hearts display multiple abnormalities in one or more factors such as excitation-contraction coupling, myofilament calcium sensitivity, fetal myosin isoform recapitulation (ie, increased β-MHC expression), and myocardial cell loss and fibrosis. Despite such abnormalities, the time courses of E_{n}(t) in each model possessing cMyBP-C was remarkably similar to healthy +/+ controls, and all highly significantly different from cMyBP-C −/− (Figure 2E). However, the E_{n}(t) waveforms for both t/t an −/− cMyBP-C models were nearly identical (Figures 2C and 2E).

The significant shortening of t_{es} in only the t/t and −/− suggests that cMyBP-C is specifically needed to sustain muscle stiffening during ejection. To test this, we transiently occluded the descending aorta in t/t to increase afterload pressure and thus t_{ao}, whereas heart rate was held constant by atrial pacing. Despite a higher afterload, t_{ao} was unaltered in both +/+ and t/t (Figure 3A) and remained shorter in the t/t hearts, indicating it was not predicated on the timing of ejection per se. We further tested whether a shortened t_{ao} varied at higher paced heart rates or calcium sensitivity (EMD 57033). Neither intervention impacted the disparity between genotypes (Figure 3B). To test if the shortened t_{ao} required was a chronic effect of the lack of cMyBP-C, we also studied young t/t mice (6 weeks of age, n=3). These young animals had preserved dP/dt_{max} (16 144±1029 mm Hg/s) and close to normal ejection fraction (43±1%), but still displayed markedly shortened elastance curves (t_{ao}=32±1 ms).
strips at pCa 4.5 was significantly enhanced in t/t compared with +/+. The administration of EMD 57033, a myosin filament calcium sensitizer, when the mice were 6 weeks old, ie, before significant LV remodeling such as myosin heavy chain isoform shift and fibrosis.

Figure 3. Temporal characteristics of LV elastance in instrumented mice. A, Time to Ees was consistently earlier in the t/t (closed symbols) compared with +/+ (open symbols) over various afterload conditions. Each symbol type refers to a single heart, and each occurrence of the symbol to a single measurement in that heart. B, Time of Ees occurred earlier in the t/t compared with +/+ over conditions of increased pacing frequency. Administration of EMD 57033, a myofilament calcium sensitizer, and when the mice were 6 weeks old, ie, before significant LV remodeling such as myosin heavy chain isoform shift and fibrosis, supporting this as a more primary effect of cMyBP-C absence.

Contraction of Intact RV Trabeculae
To test whether shortening of systolic stiffening was a feature of the myocardium and not only the intact chamber, RV trabeculae were studied under conditions of isometric contracture. The time to peak tension was shorter in t/t versus +/+ across various pacing rates (Figures 4A through 4C), with significant slowing of tension decay rate. The latter is consistent with reduced chamber –dP/dtmin (and relaxation time constant) in these hearts. It could in part be due to the higher V3 myosin isoform content.17,34 Interestingly, the absolute magnitude of systolic force development was similar at physiological rates, and even greater than +/+ controls at slower rates (Figure 4D).

Skinned Myocardial Strips
Unloaded shortening velocity (Vmax) in skinned myocardial strips at pCa 4.5 was significantly enhanced in t/t compared with +/+ (Figure 5). A similar enhancement of Vmax has been reported in isolated rat cardiac myocytes that had cMyBP-C removed.21 This result is particularly notable in light of the increased V3/V1 myosin isoform ratio in the t/t, which would be expected to lower Vmax.34 Thus, the absence of cMyBP-C in t/t must itself significantly reduce internal sarcomere loading as previously suggested.21

The isometric tension-pCa relationship for t/t displayed a significantly lower tension at relaxation and submaximal pCa ≥6 (Figure 6A). Maximum developed tension was greater in t/t (30.5±2.7 mN/mm², n=11) versus +/+ (18.6±1.9 mN/mm², n=16), consistent with the observations of higher developed tension in the t/t intact trabeculae at low pacing frequencies (Figure 4D). The calcium sensitivity (pCaCa) of t/t (5.57±0.06) and +/+ (5.62±0.03) skinned strips were not significantly different; however, the Hill coefficient was greater in t/t (4.55±0.51 versus 3.09±0.20).

Figure 4. Characteristics of intact RV trabeculae. A, Representative transients of normalized isometric tension illustrate the early time to peak tension followed by slow decay of tension in the t/t compared with +/+. B, Time-to-peak tension was shorter in the t/t under various pacing frequencies. C, Time to 90% recovery in the t/t was not different than that in +/+ at 4-Hz pacing frequency. However, at higher pacing frequencies, time to 90% recovery was longer in the t/t. D, Peak tension at 4 Hz was significantly higher in the t/t, and the force-frequency relationship was blunted in the t/t compared with +/+. n=5 t/t, 8 +/+.
Figure 5. Unloaded shortening velocity (V_{max}) in skinned strips. Times to force redevelopment for (A) +/+ and (C) t/t were identified (—) over various changes in muscle length (ML) using a second-order polynomial fit (bold line) to the recorded force. V_{max} was calculated for (B) +/+ and (D) t/t as the slope of the ML vs times to force development (∆). E, V_{max} was significantly faster in the t/t compared with +/+; n=9 t/t, 13 +/+.

The viscous modulus at pCa 8, ie, crossbridge-independent measure of viscosity, was also significantly lower in the t/t compared with +/+ at all frequencies examined up to 100 Hz except in the range 6 to 12 Hz (Figure 6D). These mechanical properties under relaxed conditions suggest that crossbridge-independent elastic load was significantly reduced in the t/t myocardium at all frequencies. In addition, the crossbridge-independent viscous load was significantly reduced in the t/t myocardium for those frequencies greater than 12 Hz, including those corresponding to harmonics of the cardiac cycle, ie, multiples of 9 Hz.

The measure of elastic modulus at pCa 4.5 includes crossbridge-dependent elasticity and was comparable between t/t and +/+ at frequencies up to 15 Hz (Figure 6E). The viscous modulus at pCa 4.5 was comparable between t/t and +/+ up to 7 Hz, but was greater in the t/t for the range 8 to 70 Hz (Figure 6F). These results recorded under maximum calcium activated conditions suggest that the crossbridge-dependent elastic and viscous loads in the t/t myofilaments were at least as high as that in +/+.

Discussion

Prior studies of the effects of a deficient abundance (t/t) or absence (−/−) of cMyBP-C on mouse LV structure and function have revealed a dilated cardiomyopathy with increased LV wall thickness, increased chamber dimensions, reduced fractional shortening, delayed relaxation, fibrosis, and myocyte disarray. Intriguingly, the early maximal rate of pressure rise was unaltered in both models, an unusual feature for hearts with dilated failure. Furthermore, recent analysis of cMyBP-C−/− skinned cardiomyocytes revealed normal peak tension and enhanced power production compared with controls. Although this suggested that cMyBP-C normally acts to impede myofilament work production, it left open the question of how lack of the protein could result in a depressed dilated heart. The present study helps reconcile these observations by revealing a novel mechanism to explain and to explain this chamber dysfunction, and highlighting a striking disparity in the impact of cMyBP-C on early versus later phases of systole. cMyBP-C appears critically important less for initiating force develop-
ment than for sustaining force and muscle stiffening so that systole can be normally extended throughout the critical period of ejection. Without it, chambers can only eject for a very short duration thereby depressing cardiac output. In the intact chamber, severe lack or absence of cMyBP-C led to striking abbreviation of the time to peak elastance (t\text{es}), with premature relaxation starting shortly after ejection was initiated. The lack of t\text{es} prolongation at higher afterload (which delayed the onset of ejection) and shortening of t\text{es} in isometric twitches of t/t myocardium suggest this is due to an intrinsic interaction between myofilaments and calcium rather than to a limitation to a given level of stress development and muscle shortening per se. The calcium transient in t/t cardiomyocytes is reportedly similar in magnitude to +/- but with significantly delayed calcium reuptake.\textsuperscript{36} Crossing mice lacking the phospholamban gene with the t/t mouse increased calcium transients and reuptake by the sarcoplasmic reticulum (SR) and improved contractile and relaxation of isolated cardiomyocytes. However, ventricular chamber function was not improved.\textsuperscript{36} The present data help explain this disparity, as abbreviated elastance kinetics in t/t hearts would impede systolic function in a mechanically loaded heart or muscle, but not necessarily in unloaded myocytes. Furthermore, loss of phospholamban itself shortens t\text{es} as recently reported,\textsuperscript{37} making it less likely that this would improve an already abbreviated systolic stiffening process in hearts lacking cMyBP-C.

Although difficult to definitively prove, the abbreviated elastance in t/t and +/- hearts appears fairly specific to cMyBP-C, in that it was not as mimicked in a broad array of models of cardiac dysfunction. Autoimmune myocarditis results in diffuse fibrosis, myocyte damage, and cardiodepression.\textsuperscript{30} MKK3-overexpressing hearts have enhanced p38 mitogen activated kinase activation, and display depressed systolic function, diastolic stiffening with fibrosis, marked increase in \(\beta\)-MHC expression, reduced SR calcium handling proteins, and other abnormalities typical of cardiac failure.\textsuperscript{31} The TnI-truncation mutation has been proposed as a model of myocardial stunning and exhibits depressed myofilament calcium sensitivity,\textsuperscript{32} whereas the desmin knockout model involves loss of an extrasarcomeric-linkage protein and exhibits hypertrophy and dilated cardiomyopathy.\textsuperscript{33} Although cardiac dysfunction is marked with each model, \(E(t)\) appears little altered. This is concordant with \(E(t)\) curve similarities in humans with varying cardiac disease conditions.\textsuperscript{38} Similarity of human and murine \(E(t)\) waveforms (adjusted for heart rate differences)\textsuperscript{22} further supports conservation of this waveshape. In our experience the \(E(t)\) relation for the t/t and +/- hearts is quite unusual, suggesting a more specific role of cMyBP-C to this process.

Multiple underlying abnormalities were revealed in the present study that could explain this phenomenon. First, t/t skinned myocardium had faster unloaded shortening velocity. These results, in addition to the greater contractile power and rate of force redevelopment reported in +/- cardiomyocytes,\textsuperscript{22} support an important intrinsic role of cMyBP-C to provide an internal load on the sarcomere.\textsuperscript{11,21} Such increased velocities and rates would be anticipated to underlie the normal rate of LV pressure generation in the otherwise failing t/t and +/- hearts and the earlier time to peak tension in the t/t trabeculae. Second, both tension and stiffness of t/t skinned myocardium was much lower at submaximal calcium. Thus, as calcium concentration declined in t/t myofilaments, one would expect a more rapid decline in absolute force or stiffness independent of the calcium sensitivity of normalized force or stiffness.

It should be noted that the higher V3 myosin fraction in t/t would, if anything, have predicted a more prolonged systolic period and reduced unloaded shortening velocity.\textsuperscript{34} The present study provides the first direct evidence that crossbridge-independent elastic internal load (Figure 6C) and crossbridge-independent viscous internal load (Figure 6D) are indeed significantly reduced in myofilaments lacking cMyBP-C. Furthermore, we demonstrate how this is translated to altering chamber elastance kinetics and thereby function.

In addition to highlighting the significance of cMyBP-C in abnormal cardiac function in mice, the current study may be relevant to our understanding mechanisms that underlie some forms of human familial hypertrophic cardiomyopathy (FHC). The majority of the FHC-causing mutant alleles for cMyBP-C code for a truncation at the C-terminus,\textsuperscript{36} where the binding sites for myosin, for titin, and for other cMyBP-C subdomains reside.\textsuperscript{2,4} The sarcomeric incorporation of cMyBP-C missing these structurally important binding sites is so far not definitive: some truncated cMyBP-C compete successfully with normal cMyBP-C and are incorporated into the sarcomere to act possibly as poison peptides,\textsuperscript{15,16,18} and other truncated forms of cMyBP-C, like that in the t/t mice (Figure 1), are not effectively incorporated into the sarcomere.\textsuperscript{23,40–42} In some human FHC patients carrying an allele that truncates cMyBP-C, the gene-coded cMyBP-C has not been detected and a causative poison peptide mechanism would therefore be highly unlikely.\textsuperscript{40–42} It is possible then that some of the functional attributes due to a deficient incorporation of normal cMyBP-C, as presented in the current study, may underlie the development of FHC in these patients.

In conclusion, the abbreviated ejection phase in the t/t and +/- mice, which lacked cMyBP-C, was observed as the inability of the LV to maintain LV pressure and elastance necessary during systolic ejection (Figure 2). Analysis of isovolumic contractile function, as frequently characterized in mice, would not have identified this primary defect in contractile mechanics. The data from the working hearts as well as from the isolated trabeculae and skinned strips presented in the current study strongly suggest that, in addition to other possible functions, cMyBP-C provides mechanical stability to the myofilament lattice in such a manner as to significantly influence the transmission of force across the sarcomere and sustain systolic stiffening. A deficient incorporation of cMyBP-C therefore may reduce tension and stiffness of sarcomeres to such a degree that would lead to the disruption of myocytes and trigger a progression to cardiomyopathy.

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