Cardiac Myosin-Binding Protein C Is Required for Complete Relaxation in Intact Myocytes

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Abstract—The role of cardiac myosin-binding protein C (cMyBP-C) in cardiac contraction is still not fully resolved. Experimental ablation of cMyBP-C by various means resulted in inconsistent changes in Ca\(^{2+}\) sensitivity and increased velocity of force of skinned preparations. To evaluate how these effects are integrated in an intact, living myocyte context, we investigated consequences of cMyBP-C ablation in ventricular myocytes and left atria from cMyBP-C knock-out (KO) mice compared with wild-type (WT). At 6 weeks, KO myocytes exhibited mild hypertrophy that became more pronounced by 30 weeks. Isolated cells from KO exhibited markedly lower diastolic sarcomere length (SL) without change in diastolic Ca\(^{2+}\). The lower SL in KO was partly abolished by the actin-myosin ATPase inhibitors 2,3-butanedione monoxime or blebbistatin, indicating residual actin-myosin interaction in diastole. The relationship between cytosolic Ca\(^{2+}\) and SL showed that KO cells started to contract at lower Ca\(^{2+}\) without reaching a higher maximum, yielding a smaller area of the phase-plane diagram. Both sarcomere shortening and Ca\(^{2+}\) transient were prolonged in KO. Isolated KO left atria exhibited a marked increase in sensitivity to external Ca\(^{2+}\) and, in contrast to WT, continued to develop twitch force at low micromolar Ca\(^{2+}\). Taken together, the main consequence of cMyBP-C ablation was a defect in diastolic relaxation and a smaller dynamic range of cell shortening, both of which likely result from the increased myofilament Ca\(^{2+}\) sensitivity. Our findings indicate that cMyBP-C functions as a restraint on myosin-actin interaction at low Ca\(^{2+}\) and short SL to allow complete relaxation during diastole. (Circ Res. 2007;101:928-938.)

Key Words: cardiac myocytes  ■ contraction  ■ familial hypertrophic cardiomyopathy
■ hypertrophy  ■ transgenic mice

Cardiac myosin-binding protein C (cMyBP-C) is located in the A-band of the cardiac sarcomere, where it interacts with myosin, actin, and titin.\(^1-5\) It consists of 11 modules labeled C0 to C10 from the N to the C terminus. It is assumed to play important structural and functional roles in health and disease (for reviews, see\(^6-10\)). Interest in cMyBP-C has intensified since the discovery that mutations in the human gene are frequently involved in familial hypertrophic cardiomyopathy (FHC).\(^11-14\) Most of them should produce C-terminal truncated cMyBP-Cs\(^14,15\) that are unstable in cardiac myocytes and undetectable in myocardial tissue of patients.\(^16-18\) A recent study suggests that they are rapidly and quantitatively degraded by the ubiquitin-proteasome system.\(^19\)

cMyBP-C can be phosphorylated at 3 different sites by cAMP-regulated protein kinase and a Ca\(^{2+}\)-calmodulin kinase bound to the thick filament (for reviews, see\(^6-10\)). Lower amount of phosphorylated cMyBP-C has been found in human failing heart and atrial fibrillation,\(^20,23\) and during low-flow ischemia.\(^22\) Recent data suggest that cMyBP-C phosphorylation is cardioprotective.\(^23\)

The regulatory role of cMyBP-C on contraction is still controversial, although there is a general agreement that cMyBP-C acts as an internal load. Removal of cMyBP-C can increase the velocity of shortening, force output, and force redevelopment in skinned preparations.\(^5,24-27\) Addition of N-terminal fragments of cMyBP-C in skinned myocytes activated force production in the absence of Ca\(^{2+}\).\(^28\) In 2 different cMyBP-C knock-out (KO) mice, fractional shortening was reduced,\(^29,30\) whereas no change in dP/dt\(_{\text{max}}\) was found.\(^30\) The maximum Ca\(^{2+}\)-activated force (F\(_{\text{max}}\)) was unaltered in skinned myocytes partially depleted in cMyBP-C\(^24\) or...
isolated from KO mice. The results of ablation or partial extraction of cMyBP-C on Ca\textsuperscript{2+} sensitivity of skinned myocytes or cardiac preparations varied among different studies. The aim of the present study was to evaluate how these alterations are integrated in an intact, living myocyte context, in which at least some of the compensatory changes for the absence of cMyBP-C remain and can be quantified. For this purpose, we have used intact, freely-suspended adult ventricular myocytes isolated from cMyBP-C WT and KO mice. Both sarcomere shortening and Ca\textsuperscript{2+} transients were measured. In addition, the relationship between external Ca\textsuperscript{2+} and force was measured in intact loaded left atrial muscles. The amounts of major myofilament and Ca\textsuperscript{2+}-handling proteins were determined to detect potential compensatory changes.

**Materials and Methods**

For an extended description see supplement data (available online at http://circres.ahajournals.org).

**Animals**

The cMyBP-C KO mice were generated in a blackswiss background as previously described.

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**Sarcomere Shortening and Ca\textsuperscript{2+} Transients Measurements in Intact Ventricular Myocytes**

Ventricular myocytes were isolated from KO and WT hearts as previously described. Cells were incubated in the IonOptix solution (in mmol/L: 135 NaCl, 4.7 KCl, 0.6 KH\textsubscript{2}PO\textsubscript{4}, 0.6 Na\textsubscript{2}HPO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 1.25 CaCl\textsubscript{2}, 20 glucose, 10 Hepes, pH 7.46) containing 1 µmol/L Fura-2-AM for 20 minutes. Sarcomere shortening and Ca\textsuperscript{2+} transients of intact myocytes were simultaneously assessed on field stimulation (1 Hz with 4 ms duration, 10 V) using a video-based sarcomere length (SL) detection system (IonOptix Corporation) at room temperature. Cells were alternatively excited at 340 and 380 nm with 510 nm emissions using the hyper-switch dual excitation light source. The F340/F380 ratio was used as an index of cytosolic Ca\textsuperscript{2+} concentration.

**Response of Isolated Left Atrial Muscles to External Ca\textsuperscript{2+}, Determination of Myosin-Heavy Chain Content, Western Blot, and Immunofluorescence Analyses**

Methods are detailed in the online data supplement.

**Results**

**Contribution of Myocyte Hypertrophy to Ventricular Hypertrophy in cMyBP-C KO Mice**

The absence of cMyBP-C in KO mice was verified by immunofluorescence in isolated myocytes (Figure 1A). Ven-
tricular-to-body-weight ratio was 29% and 44% greater in KO than in WT at 6 and 30 weeks, respectively (Figure 1B). Cell length and width of ventricular myocytes were measured in the presence of 10 mmol/L 2,3-butanedione monoxime (BDM). KO myocytes were 18% and 13% longer than WT myocytes at 6 and 30 weeks, respectively, and wider only at 30 weeks by 22% (Figure 1C). Cell area, calculated as cell length \times width, was significantly greater at 6 and 30 weeks (+13% and +28%, respectively), indicating that ventricular hypertrophy in the KO mice was mainly the result of myocyte hypertrophy.

Impairment of Shortening Kinetics in cMyBP-C KO Myocytes

Sarcomere shortenings of intact WT and KO myocytes were measured in 6-week-old (mild hypertrophy) and 30-week-old (larger degree of hypertrophy) mice (Figure 1D; Table). Diastolic SL was markedly lower in KO than in WT myocytes at both ages. In fact, SL in KO in diastole was identical to SL in WT in systole, ie, sarcomere shortening in KO started where it ended in WT. Whereas maximal sarcomere shortening did not change between KO and WT, the kinetics of both sarcomere shortening and relengthening were slower in KO. This worsened with age. Thus, shortening velocity in KO was 34% and 56% lower and relengthening velocity 33% and 49% lower at 6 and 30 weeks, respectively.

Interestingly, whereas no correlation was found between diastolic SL and sarcomere shortening in WT (Figure 2A), a slight, significant negative correlation was found in KO. Systolic SL showed the expected negative correlation with sarcomere shortening in both groups at both ages (Figure 2B). However, the slopes were significantly lower in KO and lower with age in both groups. A similar negative correlation was found between systolic SL and relengthening velocity with even more pronounced difference in slope between KO and WT (Figure 2C).

Compensatory Changes in cMyBP-C KO Mice

The amount of key myofilament and Ca\(^{2+}\)-handling proteins was determined to investigate potential adaptations to the ablation of cMyBP-C. MHC isoforms were analyzed by long electrophoresis in a polyacrylamide gel containing glycerol (Figure 3A). A significant shift from \(\alpha-\) to \(\beta\)-MHC was observed in KO at 6 weeks and to a lesser extent at 30 weeks. Thus, the amount of \(\beta\)-MHC was 5-fold and 3-fold higher in KO than in WT at 6 and 30 weeks, respectively. This finding was confirmed by Western blot with a \(\beta\)-MHC-specific antibody (Figure 3B). The amount of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) and phospholamban (PLB) was identical in KO and WT. However, the level of both phosphorylated forms of PLB was 2- to 3-fold higher in KO than in WT at 30 weeks, but not at 6 weeks of age (Figure 3B). Interestingly, the amount of Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) was 34% lower in KO than in WT (Figure 3C).

Residual Diastolic Actin-Myosin Interaction in cMyBP-C KO Myocytes

To test whether lower diastolic SL is explained by active cross-bridge cycling in KO, the effect of 10 mmol/L BDM, a chemical phosphatase that inhibits cross-bridge cycling,\(^3\) in the presence of low external Ca\(^{2+}\) (0.0125 mmol/L) was examined. Whereas BDM did not change diastolic SL in WT, it increased it by 4.7% in KO (Figure 4A). We then investigated whether the BDM effect is also observed in the complete absence of extracellular Ca\(^{2+}\) by adding 1 or 10 mmol/L EGTA. Lowering external Ca\(^{2+}\) from 1.25 to 0.0125 mmol/L induced cell arrest, but did not change diastolic SL in both groups (Figure 4B and 4C). Of note, cell arrest developed 2-fold slower in KO (Figure 4B and 4D). EGTA 10 mmol/L did not change diastolic SL in both groups. Whereas BDM did not significantly change diastolic SL in WT, it significantly increased it in KO (Figure 4C). Time from BDM injection to plateau tended to be longer in KO (521±66 versus 380±54 s in WT, \(P>0.05\)). Whereas 10 or 30 mmol/L BDM did not fully relax KO cells, 100 mmol/L BDM returned the diastolic SL in KO to WT baseline value (supplemental Figure I). Similar effects were obtained in KO with 10 \(\mu\)mol/L blebbistatin, another inhibitor of actin-activated myosin ATPase\(^{16}\) (1.73±0.02 versus 1.69±0.03 \(\mu\)m in EGTA, \(n=4\)).

Alteration of Ca\(^{2+}\) Transient Kinetics but Not Diastolic Ca\(^{2+}\) in cMyBP-C KO Myocytes

We then investigated whether lower diastolic SL and slower sarcomere shortening in KO are associated with changes in

| Table. Sarcomere Shortening in Intact cMyBP-C KO and WT Cardiac Myocytes |
|--------------------------|-------|-------|-------|-------|
|                         | WT6   | KO6   | WT30  | KO30  |
| Diastolic SL, \(\mu\)m  | 1.82±0.01 | 1.70±0.01*** | 1.74±0.01 | 1.64±0.02*** |
| Systolic SL, \(\mu\)m   | 1.69±0.01 | 1.59±0.01*** | 1.64±0.02 | 1.54±0.02*** |
| Sarcomere shortening, %  | 7±1   | 7±1   | 6±1   | 6±1   |
| Shortening velocity, \(\mu\)m/s | -2.86±0.14 | -2.14±0.09*** | -2.51±0.24 | -1.61±0.11*** |
| Time to peak shortening, ms | 96±2   | 106±3** | 95±2  | 137±5*** |
| Relengthening velocity, \(\mu\)m/s | 1.93±0.13 | 1.45±0.09*** | 1.68±0.18 | 1.13±0.10** |
| Time to 50% relengthening, ms | 60±2   | 73±3*** | 57±3  | 86±4** |
| No. of mice/cells        | 17/110 | 24/132 | 9/47  | 15/71 |
| Age of the mice, wks     | 6.6±0.2 | 6.6±0.1 | 28.7±0.7 | 27.9±0.7 |

Measurements were performed under 1-Hz electrical stimulation, 10V, in the presence of external Ca\(^{2+}\) of 1-1.25 mmol/L. Values are mean±SEM.

\(*P<0.05, **P<0.01, and ***P<0.001 vs WT, Student t test.\)
diastolic Ca\textsuperscript{2+} and Ca\textsuperscript{2+} transient kinetics, respectively. Sarcomere shortenings and Ca\textsuperscript{2+} transients were simultaneously measured in Fura-2-AM-preincubated cells from 6-week-old mice. Diastolic Ca\textsuperscript{2+} was unaltered in KO (Figure 5A through 5C). Whereas the increase in time-to-peak shortening was approximately matched by a similar increase in time-to-peak Ca\textsuperscript{2+} (27%), the slowing in relengthening (34%) corresponded to only a minor increase in time-to-50% Ca\textsuperscript{2+} decay (11%). In contrast, no alteration of the maximal amplitude of both sarcomere shortening and Ca\textsuperscript{2+} transients was detected in KO (supplemental Figure IIA). In contrast, KO cells, which were not preincubated with Fura-2 exhibited a higher sarcomere shortening at 0.5 mmol/L external Ca\textsuperscript{2+} compared with WT (supplemental Figure IIB). This divergence may relate to the 4-fold lower sarcomere shortening at 0.5 mmol/L external Ca\textsuperscript{2+} in the presence of Fura-2 (0.9% versus 3.7% without Fura-2), suggesting that, under our conditions, Fura-2 acted as a Ca\textsuperscript{2+} buffer masking differences in response to low external Ca\textsuperscript{2+}.

Altered Relation Between Sarcomere Length and Cytosolic Ca\textsuperscript{2+} in Intact KO Myocytes

To get a more detailed view of the relation between sarcomere shortening and cytosolic Ca\textsuperscript{2+} we assessed the phase-plane diagrams as previously described for rat myocytes.37
Figure 3. Determination of protein levels in mouse ventricles. A, Representative SDS gel and quantitative analysis of α- and β-myosin heavy chains (α/β-MHC) in left ventricles from 6- and 30-week-old cMyBP-C KO and WT mice (WT6, KO6, WT30, KO30). Values are mean±SEM, *P<0.05 vs WT, Student t test. Number of animals is indicated in the bars. B, Typical examples and quantitative analysis of Western blots performed on ventricular proteins. Proteins (20 μg) were subjected to 10% to 15% SDS-PAGE and transferred to nitrocellulose or PVDF membranes. Western blots were stained with the antibody directed against the indicated proteins. Values are normalized to CSQ and are expressed as mean±SEM performed on 6 to 9 different samples, *P<0.05 vs WT, Student t test. C, Representative Western blot stained for NCX (Na⁺/Ca²⁺ exchanger) and CSQ and quantitative analysis of ventricular NCX level normalized to CSQ in 6- to 9-week-old WT and KO mice. Values are expressed as mean±SEM of 12 different samples, *P<0.05 vs WT, Student t test.
This analysis revealed a counter-clockwise loop (shortening proceeded upwards), which showed 3 abnormalities in KO (Figure 5D through 5F): a shift to lower SL, a start of sarcomere shortening at lower Ca\textsuperscript{2+}, and a smaller area of the loop. Whereas the shift to lower SL recapitulates the effect of alkalosis or pharmacological Ca\textsuperscript{2+} sensitization,\textsuperscript{37,38} no increase in maximal shortening or leftward shift of the relengthening phase was observed in KO.

Figure 4. Measurement of diastolic sarcomere length (SL) in intact myocytes under 1-Hz electrical stimulation. A, Diastolic SL measured in 1.25 mmol/L Ca\textsuperscript{2+} or in 0.0125 mmol/L Ca\textsuperscript{2+} plus 10 mmol/L BDM in intact myocytes isolated from 6-week-old WT and KO mice. Values are mean±SEM. **P<0.01 vs Ca\textsuperscript{2+} 1.25 mmol/L, Student t test. B, Representative SL measurements obtained in WT and KO cMyBP-C myocytes in the following conditions (in mmol/L): Ca\textsuperscript{2+} 1.25, Ca\textsuperscript{2+} 0.0125, EGTA 10, EGTA 10 plus BDM 30. C, Diastolic SL measurements performed as in B. Conditions are indicated below the bars. Values are mean±SEM, **P<0.01 vs EGTA, Student t test. D, Time from the last normal twitch to cell arrest induced by lowering external Ca\textsuperscript{2+} concentration. Values are mean±SEM, *P<0.05 vs WT, Student t test. Number of cells is indicated in the bars.
Altered Response of Isolated KO Left Atria to External Ca\(^{2+}\)

To investigate whether the changes in KO myocyte shortening translate into altered contraction of intact loaded muscle preparations, isolated, electrically paced (1 Hz) left atria were challenged with different external Ca\(^{2+}\) concentrations (Figure 6A and 6B). When a Ca\(^{2+}\)-free Tyrode’s solution was applied (calculated external Ca\(^{2+}\) = 0.002 mmol/L), active twitch force quickly ceased in WT, but continued at a reduced steady state for >5 minutes in KO. Stepwise increases in external Ca\(^{2+}\) concentrations reactivated twitch force in WT and revealed a significantly increased sensitivity to external Ca\(^{2+}\) in KO (EC\(_{50}\) 1.21±0.17 versus 2.55±0.19 mmol/L in WT, P<0.01). Moreover, cMyBP-C ablation was also associated with a trend toward a reduced slope of the external Ca\(^{2+}\)-force relationship as indicated by a slight, not significant decrease in the Hill coefficient (2.9±0.7 versus 3.7±0.8 in WT, n=4, P>0.05). These data indicate that, under physiological concentrations of external Ca\(^{2+}\), KO myocytes contract at more than half-maximal force.

Discussion

The precise role of cMyBP-C remains incompletely understood, but the prevailing evidence suggests that it acts as an internal load on actin-myosin interactions. The present data support this interpretation by showing that cMyBP-C abrogation is associated with a marked decrease in diastolic SL in unloaded myocytes and a pronounced increase in the sensitivity to external Ca\(^{2+}\) in isometrically contracting atria. The fact that these changes were already seen in young age and did not go along with changes in intracellular diastolic Ca\(^{2+}\) concentrations support the notion that they are primary consequences of cMyBP-C ablation.

The decrease in diastolic SL in KO from ~1.8 \(\mu\)m to ~1.7 \(\mu\)m could be the consequence of a reduction in forces restoring SL or an increase in forces shortening SL against the restoring force, ie, increased cross-bridge cycling. The latter is more likely for several reasons. First, in skinned KO myocytes passive tension and its regulation by PKA were unchanged.\(^{31}\) This argues against relevant changes in titin, which is also the major component of restoring forces in cardiac myocytes. Second, the difference in SL between KO and WT was sensitive to inhibitors of myosin ATPase (BDM or blebbistatin). This indicates that the diastolic SL in KO is, at least to an important part, attributable to residual cross-bridge cycling. A similar conclusion was recently drawn in a study evaluating the consequences of a troponin I mutation associated with restrictive cardiomyopathy.\(^{39}\) Finally, the former evidence that cMyBP-C acts as an internal load on contraction\(^{24-27}\) also supports the notion that the reduced resting SL in the absence of cMyBP-C is attributable to the removal of a restraint.

Residual crossbridge cycling in diastole in myocytes lacking cMyBP-C in the absence of an increase in diastolic Ca\(^{2+}\) could either indicate a Ca\(^{2+}\)-independent mechanism\(^{39}\) or be the consequence of the increased myofilament Ca\(^{2+}\) sensitivity or both. Several arguments favor the second hypothesis. First, a decreased diastolic cell-length has been observed with alkalosis or pharmacological Ca\(^{2+}\) sensitizers.\(^{37,38}\) Second, the phase-plane diagrams revealed that KO myocytes started to shorten at lower cytosolic Ca\(^{2+}\) than WT. Third, loaded KO left atria still exhibited active twitch force in nominally 0.002 mmol/L external Ca\(^{2+}\) whereas WT did not, and the EC\(_{50}\) for external Ca\(^{2+}\) to stimulate force was more than 2-fold lower than in WT. Preliminary results indicate that the amplitude of L-type Ca\(^{2+}\) current was similar in KO and WT myocytes suggesting that the higher sensitivity of KO atria to external Ca\(^{2+}\) is independent of increased Ca\(^{2+}\) influx (L. Pohlmann, M. Kruse, O. Pongs, E. Eschenhagen, L. Carrier, unpublished data, 2007). However, in contrast to classical Ca\(^{2+}\) sensitization, no increase in maximal shortening or leftward shift of the relengthening phase was observed in KO (Figure 5). These data suggest that the ablation of cMyBP-C promotes myofilament Ca\(^{2+}\) response predominantly at low, diastolic Ca\(^{2+}\), without effect at high Ca\(^{2+}\).

This interpretation is well compatible with 2 recent studies on skinned myocyte Ca\(^{2+}\) sensitivity. Whereas earlier studies had reported mixed effects of extraction or ablation of cMyBP-C on Ca\(^{2+}\) sensitivity in skinned preparations (increase,\(^{31,32}\) no change,\(^{40}\) or decrease\(^{25,26}\)), the newer data on skinned KO preparations demonstrated a lower Hill coefficient of the pCa-force relation, indicating a reduced cooperativity of the myofilament activation.\(^{27,31}\) A potential mechanistic explanation for this finding comes from a recent study describing a radial displacement of cross-bridges away from the thick filament in the absence of Ca\(^{2+}\).\(^{41}\) These results are consistent with a model in which cMyBP-C normally acts to tether myosin cross-bridges nearer to the thick filament backbone, thereby reducing the likelihood of cross-bridge binding to actin under low Ca\(^{2+}\) concentrations.

The present results were obtained in a KO model, in which, in contrast to another model,\(^{29}\) the transcription start site of the cMyBP-C gene was eliminated.\(^{30}\) This strategy results in a complete lack of both cMyBP-C mRNA and protein, but does not exclude that our results are influenced by compensatory mechanisms. Indeed, several observations favor this notion. KO exhibited cardiac hypertrophy and a fetal gene expression program (\(^{30}\) and present study), hyperphosphorylation of PLB at older age and downregulation of NCX. One may speculate that the higher amount of the slow \(\beta\)-MHC partially compensates for the increased propensity of actin-myosin interaction in cMyBP-C KO, and it is likely that it contributes to the slower sarcomere shortening in KO.\(^{42,43}\) However, whereas myocyte dysfunction worsened in KO with age the \(\beta\)-MHC fraction decreased more than 2-fold. This argues against the idea that upregulation of \(\beta\)-MHC is a major cause of myocyte dysfunction in KO. Similarly, the markedly slower relaxation and Ca\(^{2+}\) transient decay observed in KO cells was not associated with and can therefore not be explained by a change in the amount of SERCA2 or PLB. In contrast, the higher level of both phosphorylated forms of PLB in 30-week-old KO mice suggests that the mice partially compensate the intrinsic relaxation deficit by activating a pathway that accelerates Ca\(^{2+}\) uptake into the SR and therefore hastens relaxation. Preliminary results indicate a higher density of the \(\beta\)-receptors in myocardium and a stronger inotropic effect of isoprenaline in myocytes from KO mice, and therefore support this interpretation (L. Pohl-
Figure 5. Simultaneous measurements of sarcomere shortenings and Ca\textsuperscript{2+} transients in myocytes isolated from 6-week-old cMyBP-C KO and WT mice. After preincubation of the cells with 1 μmol/L Fura-2-AM for 20 minutes, measurements were performed under 1-Hz electrical stimulation. A, Representative recordings of sarcomere shortenings and Ca\textsuperscript{2+} transients in WT and KO myocytes. B, Averaged sarcomere shortening and Ca\textsuperscript{2+} transient of 56 and 65 WT and KO cells, respectively. C, Summary data for the main parameters of sarcomere shortening and Ca\textsuperscript{2+} transient. Values are mean±SEM, **P<0.01 and ***P<0.001 vs WT, Student t test. Number of cells is indicated in the bars. D, Phase-plane diagram of SL and F340/380 ratio in averaged WT and KO cells. E, Phase-plane diagram of normalized sarcomere shortening and F340/380 ratio in averaged WT and KO cells. F, F340/380 ratios giving 5 (F\textsubscript{5}), 10 (F\textsubscript{10}) and 20% (F\textsubscript{20}) of maximum shortening in averaged WT and KO cells.
mann, T. Rau, T. Eschenhagen, L. Carrier, unpublished data, 2007). Another interesting finding was the slower disappearance of twitch force after removal of extracellular Ca\(^{2+}\) in both myocytes and left atria from KO (Figures 4 and 6). This observation points to a defect of KO to extrude Ca\(^{2+}\) and is likely related to the decreased levels of NCX (Figure 3C), to the higher Ca\(^{2+}\) sensitivity of the myofilaments that may retain Ca\(^{2+}\),39,44 or both. In any case, both alterations likely contribute to the slower sarcomere relengthening and Ca\(^{2+}\) decay in KO. It is difficult to decide to which extent the lack of cMyBP-C as such participates in the altered kinetics of shortening, but the significantly lower slope of the correlation between systolic SL and relengthening velocity in KO (Figure 2C) suggests that the absence of cMyBP-C also affects the onset and/or the velocity of relaxation. This is compatible with an inhibitory effect of cMyBP-C on cross-bridge cycling and increased Ca\(^{2+}\) sensitivity in KO.

Residual cross-bridge cycling in diastole, incomplete relaxation, and increased Ca\(^{2+}\)-sensitivity of the myofilaments are expected to translate into diastolic dysfunction, basal hypercontractility, and increased energy expenditure, all of which are typical features of FHC.45–47 Indeed, the hemodynamic measurements in the same KO mice have revealed decreased dP/dt\(_{\text{min}}\) and increased Tau, as well as an increased LV end-systolic meridional wall stress in the basal state.30 On the other hand, cMyBP-C ablation was associated with a reduced absolute response to increasing external Ca\(^{2+}\) (from 1.8 to 6.4 mmol/L) in intact atria (Figure 6), a reduced length-dependent activation in skinned myocytes,31 and a blunted response to dobutamine in vivo.30 Taken together, these data indicate that the physiological role of cMyBP-C is to increase the dynamic range of myofilament responses mainly by allowing complete relaxation under diastolic conditions. A problem with this model is that, as recently pointed out by de Tombe,8 the substantial contractile activation in diastole should be incompatible with life. Whereas this is obviously not the case in mice, the same does not necessarily apply to human in which cardiac function depends much more on regulation of contractile force. Indeed, a homozygous Q76ter mutation, which is expected to produce a functional cMyBP-C KO, has been shown to be associated with massive hypertrophy and sudden death by 9 months of age in a FHC patient.14

Taken together, our results suggest that cMyBP-C acts as an internal load that tethers myosin heads to the thick-filament backbone and prevents force generation in diastole. Its absence causes cross-bridge cycling at low diastolic Ca\(^{2+}\), higher myofilament Ca\(^{2+}\) sensitivity with a reduced cooperativity, hypercontractility in the basal state, and reduced dynamic range of contraction. These changes are well compatible with the phenotype of human FHC and, in human, are likely to occur even when the amount of cMyBP-C is discretely reduced as may result from haploinsufficiency.

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**Disclosures**

None.

**References**


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DATA SUPPLEMENT

EXPANDED MATERIALS AND METHODS

The investigation conforms to the guide for the care and use of laboratory animals published by the NIH (Publication No. 85-23, revised 1985).

Animals

The cMyBP-C KO mouse model was based upon the targeted deletion of exons 1-2 of the mouse cMyBP-C gene, which also includes the transcription initiation site.¹ Male and female WT and KO mice were studied at 6-7 and 28-29 weeks of age, which were defined as the 6 wks (WT6 and KO6) and 30 wks (WT30 and KO30) groups, respectively.

Ventricular myocyte preparation

Mice were sacrificed by cervical dislocation in light CO₂ anesthesia. Hearts were mounted on a temperature-controlled perfusion system, perfused with a Ca²⁺-free solution (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 30 mM taurine, 5.5 mM glucose, 10 mM 2,3-butanedione monoxime (BDM), 10 mM HEPES, pH 7.46) at 37°C, and then digested for 9-10 min in the Ca²⁺-free solution containing 0.1 mg/mL Liberase Blendzyme (Roche Diagnostics). The ventricles were then cut into pieces to dissociate single cells. Ca²⁺ was stepwise reintroduced to a final concentration of 0.5-1 mM.
**Sarcomere shortening and Ca$^{2+}$ transients measurements in intact ventricular myocytes**

Morphological and functional criteria for using a ventricular myocyte for the experiments were: i) rod-shaped, ii) no membrane blebs, iii) no hypercontractile zones, iv) no spontaneous contractions, and v) stable contraction amplitude at 1 Hz. Cells were incubated in the IonOptix solution (135 mM NaCl, 4.7 mM KCl, 0.6 mM KH$_2$PO$_4$, 0.6 mM Na$_2$HPO$_4$, 1.2 mM MgSO$_4$, 1.25 mM CaCl$_2$, 20 mM glucose, 10 mM Hepes, pH 7.46) containing 1 µM Fura-2-AM for 20 min, then rinsed for 10 min in the IonOptix solution. In some cases indicated in the legend, cells were incubated without Fura-2-AM in IonOptix solution containing different concentrations of Ca$^{2+}$ with or without Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), 10 mM 2,3-butanedione monoxime (BDM, Sigma) or 10 µM 1-phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo[2.3-b]-7-methylquinolin-4-one (blebbistatin, Sigma). Sarcomere shortening and Ca$^{2+}$ transients of intact myocytes were simultaneously assessed upon field stimulation (1-Hz with 4-ms duration, 10 V) using a video-based sarcomere length (SL) detection system (IonOptix corporation) at room temperature (RT). Cells were alternatively excited at 340 and 380 nm with 510 nm emissions using the hyper-Switch dual excitation light source from the IonOptix system. The F340/F380 ratio was used as an index of cytosolic Ca$^{2+}$ concentration.

**Response of isolated left atrial muscles to external Ca$^{2+}$**

Experiments were performed at 37°C as described previously under 1-Hz stimulation. Muscle length was adjusted to its half-maximal twitch force in a modified Tyrode’s solution containing 1.8 mM Ca$^{2+}$, and then Ca$^{2+}$ was removed by rinsing twice the upright organ bath with 30 ml of a Ca$^{2+}$-free Tyrode solution (calculated external Ca$^{2+}$ 0.002 mM), which should give a external Ca$^{2+}$ concentration of about 0.002 mM. The effect of increasing Ca$^{2+}$ concentrations (0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 4.0, 4.8, 5.6, 6.4 mM) on force of contraction was measured.
Antibodies

A rabbit polyclonal anti-cMyBP-C antibody (directed against the MyBPC motif) was kindly given by C. Witt and W. Linke (Heidelberg, Germany). Mouse monoclonal anti-β-myosin heavy chain (β-MHC, M8421) and anti-α-actinin antibodies was purchased from Sigma, mouse monoclonal anti-Na⁺/Ca²⁺ exchanger antibody from Abcam (NCX1), rabbit polyclonal anti-calsequestrin antibody from Affinity Bioreagents (PA1-913), mouse monoclonal antibodies directed against total phospholamban (PLB) A1, phospho-PLB (Ser-16) PS-16 and phospho-PLB (Thr-17) PT-17 from Badrilla, and goat polyclonal antibody directed against the sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA2) N-19 from Santa Cruz. Secondary antibodies anti-rabbit IgG HRP-conjugated and anti-mouse IgG HRP-conjugated were purchased from Sigma. Secondary anti-goat IgG HRP-conjugated antibody was from Santa Cruz. Secondary anti-mouse IgG antibody conjugated with Alexa 488 and anti-rabbit IgG antibody conjugated with Alexa 546 were from Molecular probes.

Western-blot analysis

Tissues were homogenized with a polytron in 5% SDS, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 75 mM urea, 1 mM DTT at 4°C and centrifuged at 14000 rpm for 10 min. The supernatant was collected and protein concentration was determined according to Bradford. Proteins were loaded on 10-15% acrylamide/bisacrylamide (37.5:1)-SDS gels, electro-transferred onto a nitrocellulose or polyvinylidene fluoride membrane, incubated with different antibodies and detected by chemiluminescence. The dilutions of antibodies used were: NCX1 1:1000, calsequestrin 1:2500, SERCA2 1:100, MHC 1:4000, PLB A1 1:5000, Ser16-PLB 1:5000, Thr17-PLB 1:5000, anti-rabbit HRP 1:10000, anti-mouse HRP 1:5000, and anti-goat HRP 1:5000.
**Immunofluorescence analysis**

Adult mouse ventricular myocytes were plated on laminin-coated (0.01 mg/ml PBS) Labtek chambers (20,000 rod-shaped cells/ml) in MEM medium (supplemented with Hanks’ salts and 2 mM glutamine), 100 U penicillin/streptomycin, 10 mM BDM. After 2 h, medium was removed and cells were rinsed twice in PBS and fixed for 7 min at -20°C in methanol/acetone (20/80). After washing in PBS, cells were permeabilized for 1 h at room temperature (RT) in solution A (0.5% Triton X-100, 1% BSA, 10% FCS in PBS). After washing in solution B (0.5% Triton X-100, 1% BSA in PBS), cells were incubated for 45 min at RT with primary antibodies diluted in solution B (anti-cMyBP-C, dilution 1:500 and anti-α-actinin, dilution 1:200). Cells were rinsed twice in solution B and then incubated for 45 min at RT and in the dark with secondary antibodies diluted in solution B (anti-rabbit IgG Alexa 546-conjugated, dilution 1:600 and anti-mouse IgG Alexa 488-conjugated, dilution 1:600). Cells were incubated with 1 µM ToPro3 (Molecular Probes) for 10 min, rinsed three times with PBS and then fixed with Mowiol. Fluorescence was analyzed by confocal microscopy using a Zeiss confocal inverted microscope with a 40x-oil objective.

**Myosin-heavy chain content**

The composition of gel sample, gel preparation and composition, and the gel running conditions were identical to those described previously. Myosin isoform composition was determined on purified myosin stored in 50% glycerol. The solution was diluted 1:10 with sample buffer and 3-4 µl were loaded on the gel. Stacking gels contained 4% acrylamide/bisacrylamide (50:1) and 10% (vol/vol) glycerol. Separating gels contained 8% acrylamide/bisacrylamide (50:1) and 10% (vol/vol) glycerol. Gels run for 24 h at a constant voltage of 70 V in a mini-protean II gel system (Biorad) at 4°C. Gels were stained with
Coomassie blue, and quantification of α- and β-MHC amount was performed with the Image Gauge software.

**Statistical analysis**

Data are presented as mean±SEM. Statistical analyses were performed using the unpaired Student’s t-test, the one-way and two-way ANOVA and Bonferroni multiple comparisons. Spearman correlation and linear regression analyses were performed to assess the relationship between SL and fractional shortenings and between SL and relengthening velocities. Analyses were performed using a commercial software (GraphPad Software Inc). A value of $P<0.05$ was considered significant.

**References**


Supplement Figure 1. Diastolic SL (sarcomere length) measured in cMyBP-C WT (wild-type, n=6) and KO (n=4) cells in the presence of 1.5 mM external Ca$^{2+}$. The effect of 10 and 100 mM BDM was analysed in the KO cells. Values are mean±SEM, *$P$<0.05 and **$P$<0.01 vs. WT, unpaired Student’s t-test.
Supplement Figure 2. Relation between maximal sarcomere shortening or Ca$^{2+}$ transient and external Ca$^{2+}$ concentrations in cells pre-incubated (A) or not (B) with Fura-2-AM. Measurements were performed at room temperature, under 1-Hz electrical stimulation, and in the presence of different external Ca$^{2+}$ concentrations. A) Maximal sarcomere shortening (%) and maximal Ca$^{2+}$ transient (ΔF340/380 ratio) measured in 23 WT and KO myocytes isolated from 6 wks-old mice. *$P<0.05$, Two-way Anova. B) Maximal sarcomere shortening measured in intact myocytes isolated from 30 wks-old KO and WT mice. Values are mean±SEM of >26 cells, **$P<0.01$ vs. WT, Student’s t-test.