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Normal cardiac contraction in mice lacking the proline-alanine rich region and C1 domain of cardiac myosin binding protein C.

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Abstract

Cardiac myosin binding protein C (cMyBP-C) is an essential regulator of cross bridge cycling. Through mechanisms that are incompletely understood the N-terminal domains (NTDs) of cMyBP-C can activate contraction even in the absence of calcium and can also inhibit cross bridge kinetics in the presence of calcium. *In vitro* studies indicated that the proline-alanine rich (p/a) region and C1 domain are involved in these processes, although effects were greater using human proteins compared to murine proteins (Shaffer *et al.* J Biomed Biotechnol 2010, 2010:789798). We hypothesized that the p/a and C1 region are critical for the timing of contraction. In this study we tested this hypothesis using a mouse model lacking the p/a and C1 region (p/a-C1^{-/-} mice) to investigate the *in vivo* relevance of these regions on cardiac performance.

Surprisingly, hearts of adult p/a-C1^{-/-} mice functioned normally both on a cellular and whole organ level. Force measurements in permeabilized cardiomyocytes from adult p/a-C1^{-/-} mice and wild type (Wt) littermate controls demonstrated similar rates of force redevelopment both at submaximal and maximal activation. Maximal and passive force and calcium sensitivity of force were comparable between groups as well. Echocardiograms showed normal isovolumetric contraction times, fractional shortening and ejection fraction, indicating proper systolic function in p/a-C1^{-/-} mouse hearts. p/a-C1^{-/-} mice showed a slight but significant reduction in isovolumetric relaxation time compared to Wt littermates, yet this difference disappeared in older mice (7–8 months of age). Moreover, stroke volume was preserved in p/a-C1^{-/-} mice, corroborating sufficient time for normal filling of the heart. Overall, the hearts of p/a-C1^{-/-} mice showed no signs of dysfunction even after chronic stress with an adrenergic agonist.

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Disclosures
None.

Together, these results indicate that in mice the p/a region and the C1 domain of cMyBP-C are not critical for normal cardiac contraction and that these domains have little if any impact on cross bridge kinetics at least not in mice. These results thus contrast with *in vitro* studies utilizing proteins encoding the human p/a region and C1 domain. More detailed insight in how individual domains of cMyBP-C function and interact, across species and over the wide spectrum of conditions in which the heart has to function, will be essential to a better understanding of how cMyBP-C tunes cardiac contraction.

Keywords

Myosin binding protein C; sarcomere; cardiac contraction; mice; echocardiogram

1. Introduction

Cardiac myosin binding protein C (cMyBP-C) is an essential, yet incompletely understood, sarcomeric protein. The importance of cMyBP-C is underscored by the many mutations in *MYBPC3*, the gene encoding cMyBP-C, that are associated with hypertrophic cardiomyopathy (HCM) [1, 2]. Additionally, the absence of cMyBP-C in knock out animal models leads to cardiac dysfunction and remodeling [3–5], resembling many characteristics of human cardiomyopathies resulting from cMyBP-C mutations. Whereas the C-terminal domains (CTDs) of cMyBP-C interact with light meromyosin [6–8] and titin [9], the N-terminal domains (NTDs) extend away from the thick filament backbone and interact with myosin heads [8, 10, 11] and/or actin [12–14] to influence cross bridge cycling (Figure 1).

cMyBP-C appears to repress cross bridge cycling. Cardiomyocytes from cMyBP-C knock out mice display faster cross bridge cycling as evident from faster twitch kinetics [15], faster force development following a stretch [16–18] and increased shortening velocity [17, 19]. Inhibitory effects of cMyBP-C were also evident in *in vitro* motility assays, where addition of N-terminal cMyBP-C recombinant proteins caused decreased actin sliding velocity [20, 21]. Consistent with this, actin moved faster along thick filaments that lacked cMyBP-C compared to thick filament where cMyBP-C was present [22]. Interestingly, cMyBP-C NTDs can also activate cross bridges in the absence of calcium when reconstituted thin filaments were used in *in vitro* motility assays [20]. Although the significance and precise mechanisms of the ability of cMyBP-C to activate cross bridge cycling is not well understood, such activating effects may be relevant during the late phase of systole when the intracellular calcium concentration is declining. If so, activating effects of cMyBP-C could account for the observation that hearts of cMyBP-C knockout mice demonstrated a shorter time course of elastance, leading to an abbreviated duration of the ejection phase [19]. Consistent with this Tong *et al.* demonstrated the importance of cMyBP-C and its phosphorylation to maintain a rise in force while internal calcium is already declining [23]. These results demonstrate the importance of cMyBP-C for proper systolic timing. The dual effects of cMyBP-C to activate or inhibit cross bridge cycling suggest that cMyBP-C tunes contraction by multiple mechanisms.

The functional properties of cMyBP-C are generally attributed to the NTDs, i.e. the regions from C0 to C2 (Figure 1), but details on the mechanisms underlying cMyBP-C's function or the contribution of individual domains to function are largely unknown. Incubation of detergent permeabilized cardiomyocytes with NTDs of cMyBP-C demonstrated both the inhibitory and activating properties of cMyBP-C, yet the contribution of individual domains differed depending on species [24, 25]. *In vitro* motility assays comparing effects of N-terminal recombinant proteins confirmed that the proline-alanine rich (p/a) region and C1 domain have activating effects on cross bridge cycling, and that there are species-specific differences because effects were larger using human proteins compared to murine proteins [26]. Interestingly, the p/a region is not highly conserved among species. However, the percentage of proline and alanine inversely correlated with average heart rate in mammals [27]. The differences within the p/a region between species might thus be important for the regulation of contraction speed to match it to heart rates.

To determine the physiological relevance of the p/a region and C1 domain to cardiac contraction, we used a mouse model that lacked these regions [28], referred to here as p/a-C1^{-/-} mice. As originally described by Witt *et al.* [28] the mutant cMyBP-C is incorporated into sarcomeres, but homozygous animals completely lack the p/a and C1 regions. Remarkably, p/a-C1^{-/-} mice showed no signs of cardiac remodeling [28], making them an ideal model to directly study the functional significance of the p/a-C1 region without possible confounding effects resulting from cardiac remodeling. We hypothesized that if the p/a and C1 domains are essential for the function of cMyBP-C, removal of these domains would lead to faster cross bridge cycling in cardiomyocytes from p/a-C1^{-/-} mice and systolic dysfunction. Surprisingly, deletion of such a substantial section of cMyBP-C did not cause overt dysfunction in the hearts of p/a-C1^{-/-} mice. Our results indicate that the p/a region and C1 domain are not critical for cardiac function at least not in mice.

2. Methods

2.1 Animals

Treatment of all animals was in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committees of the University of California, Davis and the University of Arizona. Homozygous mice lacking the proline-alanine rich (p/a) region and the C1 domain of cMyBP-C (p/a-C1^{-/-} mice) and wild type litter mate controls (Wt) were bred at the University of California, Davis, by the Mouse Biology Program from heterozygous embryos created by Dr. Christian Witt [28]. All mice were genotyped using forward primers located in exon 6 (5'-CGCTGTGAGGTGTCTACCAA-3') and the NEO cassette (5'-GACCACCAAGCGAAACATCG-3') and a reverse primer in exon 7 (5'-GTGCGAAAGCTGATCTGAGG-3') to identify mice that are wild type (Wt), heterozygous or homozygous for the p/a-C1 deletion. Mice used for experiments were 4–5 months old.

A subset of mice received isoprenaline (ISO) to increase cardiac stress to investigate the importance of the p/a-C1^{-/-} region when there is a higher demand on the heart. 20 µg/g isoprenaline (Sigma Aldrich) was delivered subcutaneously for 5 consecutive days to evoke cardiac hypertrophy [29]. Echocardiograms were recorded immediately before and again after 5 days of isoprenaline injections. Hearts were then excised and weighed. A separate

group of control mice received saline injections of comparable volumes instead of isoprenaline.

2.2 Tissue preparation

Mice were anesthetized by isoflurane inhalation and euthanized. Hearts were quickly excised, rinsed in a Ringer's solution (containing, in mM: 100 NaCl, 24 NaHCO₃, 2.5 KCl, 1 MgSO₄*7H₂O, 1 Na₂HPO₄ and 1 CaCl₂) with protease and phosphatase inhibitors (1:100, Halt protease and phosphatase inhibitor cocktail, ThermoScientific) and gently blotted to remove excess moisture. Hearts were weighed and left ventricles and intraventricular septa were dissected and snap frozen in liquid nitrogen. Some mice received 0.5 g/L propranolol in their drinking water one week prior to euthanasia to reduce the impact of stress during handling and euthanasia.

Multicellular myocyte preparations were obtained by mechanically disrupting frozen cardiac tissue in a Relax buffer (containing, in mM: 5.95 Na₂ATP, 6.04 MgCl₂, 2.0 EGTA, 139.6 KCl, 10 imidazole, pH 7.0 with ionic strength set to 180 mM). Preparations were subsequently permeabilized at 4°C for 20 minutes with 1% Triton X-100 in Relax buffer. Multicellular preparations were used either for immunohistochemistry, force measurements or for protein analysis.

2.3 Immunohistochemistry

Cardiomyocytes obtained by mechanical disruption of frozen cardiac tissue were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for a minimum of 2 hours at 4°C with agitation. All subsequent steps were done on cell suspensions. Solutions were removed by pelleting the cells at 3000 x g. Fixed cardiomyocytes were incubated with buffer containing ammonium sulphate in order to quench the fixative. The cardiomyocytes were then incubated with 5% normal goat serum (NGS) to prevent non-specific antibody binding. Primary antibodies against cMyBP-C (C-pro, 1:6000, rabbit polyclonal[4]) and α -actinin (Sigma-Aldrich A7811, 1:600, mouse monoclonal) were diluted in Tris buffered saline with 0.05% Tween 20 (TBS-T) with 5% NGS and myocytes were incubated with the primary antibodies overnight at 4°C. After washing the cardiomyocytes with TBS-T they were incubated with species appropriate secondary antibodies (anti-rabbit AlexaFluor 488 and anti-mouse AlexaFluor 568, both diluted 1:1800, Invitrogen) for 1–2 hours at room temperature. After washing with TBS-T, cells were pelleted and mounted in a drop of antifade (Prolong® Gold antifade with DAPI, Invitrogen) on a microscope slide using forceps to pull apart large clumps of cells. A coverslip was applied and the antifade was allowed to harden. Cells were imaged using a DeltaVision Deconvolution microscope and an Olympus 60X/ 1.40, Plan Apo, IX70 objective.

Image stacks were deconvolved using softWoRx® version 5.5 (GE Healthcare). Appropriate images were flattened into a single projection using Graphic Converter X version 6.7 (Lemke Software GmbH) and the “Merge folder into one Image” command. The images were histogram stretched and false colored in Photoshop CS5.1 (Adobe) with α -actinin in red and cMyBP-C in green.

2.4 Protein analysis

Protein homogenates were prepared by treating permeabilized multicellular preparations with 10% trichloroacetic acid and then dissolving samples in 2x urea-thiourea sample buffer (in M: 8 urea, 2 thiourea, 0.05 trizma base, 0.075 DTT, 3% SDS, 0.05% Bromophenol Blue) [30].

Expression of cMyBP-C in p/a-C1^{-/-} hearts was determined by separating left ventricle homogenates on 4–15% TGX gels (Biorad) and subsequent staining of gels with Coomassie blue (Coomassie Brilliant Blue R250, Biorad). Intensity of cMyBP-C and α -actinin bands was analyzed with either Quantity One (Biorad) or Genetools (Syngene) software. cMyBP-C expression level was also determined using Western blots. cMyBP-C was detected using a 1:200 dilution of a polyclonal goat antibody (K16, Santa Cruz) and α -actinin was detected with either a mouse monoclonal (clone 4i336, Santa Cruz Biotechnology 69973, 1:5000 dilution) or a polyclonal rabbit (clone H-300, Santa Cruz Biotechnology 15335, 1:1000 dilution) antibody. Bands were visualized using ECL (enhanced chemiluminescence) (Thermo Scientific) and analyzed with the software described above. cMyBP-C expression was normalized to α -actinin expression to correct for loading differences between individual samples.

cMyBP-C and cardiac troponin I (cTnI) phosphorylation were determined using ProQ Diamond phosphoprotein stain (Invitrogen). To detect total protein, gels were stained with Coomassie Blue. Band intensity was analyzed with the software described above and phosphorylation of each protein was normalized to α -actinin expression.

Protein kinase A (PKA) phosphorylation of cMyBP-C, cTnI and cTnT was achieved by incubating permeabilized cardiomyocytes in Relax buffer with or without 100U/mL PKA (Sigma Aldrich) for 40 min. Phosphorylation was confirmed using ProQ Diamond phosphoprotein stain as described above (Supplemental figure 2). The ability of GSK3 β to phosphorylate murine cMyBP-C was tested by incubating recombinant murine C0C2 with GSK3 β for 60 minutes. Phosphorylation was confirmed by ProQ Diamond phosphoprotein stain (Supplemental figure 3).

2.5 Force measurements

Force development in permeabilized multicellular preparations was measured as described previously [25, 31]. Preparations were mounted between a force transducer (model 403A, Aurora Scientific Inc.) and a high speed motor to control length (model 315-I, Aurora Scientific Inc.). Motor position inputs were driven using a digital controller (600A Digital Controller, Aurora Scientific Inc.).

Sarcomere length was set to 2.25 μ m and force development at various Ca²⁺ concentrations was measured at 15°C by submerging the permeabilized myocytes in Ca²⁺ solutions ranging from pCa 9.0 to 4.5 (where pCa = $-\log[\text{Ca}^{2+}]$) in sample wells of an 802D permeabilized fiber apparatus (Aurora Scientific Inc.). Before each force measurement, myocytes were preconditioned in a pre-activating solution with reduced Ca²⁺ buffering. Subsequently, myocytes were transferred to activating solutions containing calcium and steady state force was recorded. Cells were then quickly slacked and rapidly re-stretched to determine the rate

of force redevelopment (k_{tr}) [25, 31]. After each Ca^{2+} activation, measurements were repeated at pCa 9.0 to determine passive force (F_{pas}) by a fast slack and re-stretch of the cardiomyocytes.

Ca^{2+} solutions were prepared using custom software described previously [25, 32] and contained (in mM): 15 EGTA, 70 MOPS, 5 ATP, 15 phosphocreatine, 1 free Mg^{2+} , 70 free Na^+ , and >10 free K^+ . Ionic strength was set to 180 mM and pH to 7.0 at 15°C. Solutions with pCa 9.0 and 4.5 were made by adding the proper amount of $CaCl_2$. Solutions with intermediate $[Ca^{2+}]$ were made by mixing pCa 4.5 and pCa 9.0 solutions in ratios to obtain desired free $[Ca^{2+}]$. A pre-activating solution was made by replacing the EGTA with HDTA in the pCa 9.0 solution.

To determine effects of phosphorylation, force measurements were repeated after 40 minute incubation (20°C) with protein kinase A (Sigma Aldrich, 100U/mL in Relax solution). Incubating permeabilized cardiomyocytes with PKA for 40 minutes at 20°C increased phosphorylation of cMyBP-C and cTnI (Supplemental figure 2). Effects of GSK3 β were determined in a separate set of cardiomyocytes by measuring force before and after incubation for 60 minutes at 20°C with active GSK3 β (Sigma Aldrich, 20 μ l in relax, total volume of 100 μ l [33]).

Ca^{2+} activated force was calculated by subtracting F_{pas} from steady state force at each Ca^{2+} concentration and normalized for cross-sectional area of the myocyte preparation (mN/mm^2). As described previously [25], maximal force development (F_{max}) was measured at saturating $[Ca^{2+}]$ (pCa 4.5) and relative force was calculated by expressing submaximal force as a fraction of F_{max} ($F_{rel} = F/F_{max}$). k_{tr} was calculated as the rate of force redevelopment at the time when half of the maximal force is redeveloped (i.e., as the $t_{1/2}$ of force redevelopment) [25, 31]. To determine the force-calcium relationship and Ca^{2+} sensitivity of force (pCa₅₀, i.e. the pCa value at which 50% of F_{max} is reached), F_{rel} was plotted against pCa and the curve was fitted to the Hill equation: $F = F_{max} / (1 + 10^{nH(pCa-pCa50)})$.

2.6 Echocardiograms

Transthoracic echocardiograms were performed on anesthetized mice (1–3% isoflurane) using a Vevo 2100 (Fujifilm VisualSonics) equipped with a MS-550S 32–56MHz transducer. Wall thickness and left ventricular (LV) diameter were recorded from short axis M-mode images. Tracings of the left ventricle on M-mode images (e.g. Figure 5) were used to calculate fractional shortening (FS: (average diastolic diameter - average systolic diameter)/ average diastolic diameter*100), stroke volume (SV, diastolic volume - systolic volume, where volume is calculated as $7.0 / (2.4 + \text{average LV diameter}) * \text{average LV diameter}$), ejection fraction (EF: stroke volume/ diastolic volume*100) and cardiac output (CO: stroke volume*heart rate/1000). Pulse wave Doppler in an apical four chamber view was performed to determine the peak velocity of blood flow into the left ventricle during the early phase (E) and atrial contraction phase (A) of diastole. Tissue Doppler at the mitral annulus was recorded to obtain peak velocities of tissue movement during early (E') and atrial contraction (A') phases of diastole. Each measure was calculated as the average value of 3–4 separate recordings per animal.

2.7 Statistics

Values are reported as mean $\pm$ standard error of the mean (SEM). Groups were compared by unpaired Student's t-test. Force redevelopment or the effect of PKA over the range of $[Ca^{2+}]$ was compared between groups by 2-way ANOVA followed by a Bonferroni post-test. $P < 0.05$ was considered significant.

3. Results

3.1 Mutant protein is expressed normally in p/a-C1^{-/-} mice

Removal of the p/a-C1 region from cMyBP-C resulted in a shortened protein that was incorporated into sarcomeres [28]. Here we used immunostaining of cardiomyocytes from both wild type and p/a-C1^{-/-} mice with antibodies against cMyBP-C and α -actinin to show that doublets of cMyBP-C were incorporated between Z-lines (Figure 2A), confirming proper incorporation of mutant p/a-C1^{-/-} cMyBP-C into sarcomeres. To determine if mutant p/a-C1^{-/-} is expressed at similar levels as wild type cMyBP-C, we quantified expression of cMyBP-C both by Coomassie gel stain and Western blot. Figure 2B shows a representative Coomassie stained gel and a Western blot probed with antibodies against cMyBP-C and α -actinin, respectively. Results demonstrated full-length cMyBP-C in wild type (Wt) mice and mutant cMyBP-C with a lower molecular weight in p/a-C1^{-/-} mice as expected for deletion of the p/a and C1 domains. Quantification of the expression of cMyBP-C relative to α -actinin demonstrated no significant difference between groups (Figure 2C), indicating that absence of the p/a-C1 region did not reduce expression of cMyBP-C in the p/a-C1^{-/-} mice.

3.2 Normal force development in p/a-C1^{-/-} cardiomyocytes

To examine how the absence of the p/a-C1 region of cMyBP-C influenced sarcomeric contraction, we measured force development in permeabilized cardiomyocytes at various calcium concentrations. Mice were treated with propranolol before euthanasia to minimize possible confounding effects of stress in response to handling. Both maximal force development (F_{max}) and passive force (F_{pas}) were comparable between groups (Table 1). Calcium sensitivity of force (represented by pCa_{50} , the calcium concentration needed to reach 50% of maximal force, Figure 3A) was also comparable, consistent with comparable phosphorylation of cTnI and cMyBP-C in p/a-C1^{-/-} and Wt mice (Figure 4).

We hypothesized that absence of the p/a and C1 regions of cMyBP-C would reduce the inhibitory effect of cMyBP-C on cross bridge cycling, therefore we determined the speed of force redevelopment following a slack and re-stretch step (i.e., k_{tr}). k_{tr} increased with increasing force development at increased calcium concentrations in both groups as expected. K_{tr} tended to be slightly higher in p/a-C1^{-/-} mice, but was significantly different only at a single pCa (pCa 5.4 Figure 3B, Table 1). Plotting k_{tr} versus force to account for force dependent differences in k_{tr} at each pCa (Figure 3C) did not reveal significant differences between p/a-C1^{-/-} and Wt myocytes.

3.3 Protein phosphorylation and response to PKA and GSK3 β

Phosphorylation sites in the M-domain of cMyBP-C are preserved in p/a-C1^{-/-} mice and can be phosphorylated by PKA *ex vivo* [28]. We used ProQ Diamond stain to determine

endogenous phosphorylation levels of sarcomeric proteins in p/a-C1^{-/-} and Wt mice. The phosphorylation level of cMyBP-C, cardiac troponin I and T (cTnI and cTnT, respectively) were comparable between p/a-C1^{-/-} and Wt mice (Figures 4A–C). To minimize the consequences of a stress response to handling and euthanasia, mice from which the hearts were used to measure force development in cardiomyocytes were treated with propranolol in the week before they were sacrificed. Phosphorylation of cMyBP-C, cTnI and cTnT were comparable between p/a-C1^{-/-} and Wt mice after treatment with propranolol (Figures 4D–F). However, phosphorylation of cMyBP-C and cTnI were decreased compared to mice not treated with propranolol (Supplemental figure 1). Force measurements were next repeated after incubation of cardiomyocytes with protein kinase A (PKA) to establish if absence of the p/a-C1^{-/-} region alters the response to the β -adrenergic pathway. Phosphorylation of cMyBP-C and cTnI were significantly increased in cardiomyocytes incubated with PKA for 40 min at 20°C (Supplemental figure 2). However, the effect of PKA on cMyBP-C and cTnI phosphorylation was similar between groups. That is, force measurements repeated after incubation with PKA showed a significant decrease in myofilament sensitivity to calcium ($pCa_{50} = 0.18 \pm 0.02$ and 0.23 ± 0.03 in p/a-C1^{-/-} and Wt, respectively) (Table 1, Figure 3A), but effects of PKA were similar in both groups, suggesting normal myofilament response to PKA in permeabilized myocytes from p/a-C1^{-/-} mice.

Recently Kuster *et al.* [33] identified a GSK3 β phosphorylation site in the p/a domain (Ser 133). Incubation of cardiomyocytes from heart failure patients with GSK3 β increased phosphorylation of Ser133 and increased the maximal rate of tension redevelopment. Incubation of recombinant murine C0C2 with GSK3 β also phosphorylated murine protein (Supplemental figure 3A), similar to human protein [33]. However, we found no effect of GSK3 β on active or passive force, calcium sensitivity of tension or ktr in myocytes incubated with GSK3 β (Supplemental figure 3B).

3.4 Normal cardiac morphology and performance of p/a-C1^{-/-} mice hearts

To assess how deletion of the p/a and C1 regions influence heart function we performed transthoracic echocardiograms on Wt and p/a-C1^{-/-} mice (Figure 5). A summary of all echocardiographic measurements is given in Table 2. Hearts of p/a-C1^{-/-} mice showed no signs of cardiac remodeling as the dimensions of the left ventricular posterior wall (LVPW) and the left ventricular internal diameter (LVID) were not significantly different from Wt hearts (Table 2). Heart weight/ body weight ratios as determined from explanted hearts were slightly but not significantly higher in p/a-C1^{-/-} mice compared to Wt (5.41 ± 0.2 versus 4.89 ± 0.2 mg/g, respectively).

If the p/a and C1 regions of cMyBP-C are essential for cMyBP-C to match contraction speed to heart rate, then the duration of systole and diastole might be disturbed in p/a-C1^{-/-} mice. However, all major parameters defining systolic function (e.g. fractional shortening (FS), ejection fraction (EF) and stroke volume (SV) were comparable between p/a-C1^{-/-} and Wt mice. Diastolic function was evaluated by pulse-wave Doppler to measure flow through the mitral valve and tissue Doppler to measure mitral valve annulus movement. The speed of the blood flow through the mitral valve was comparable in p/a-C1^{-/-} and Wt mice during both early (E) and late atrial (A) filling. Likewise, the speed with which the mitral valve annulus

moved was similar for both groups. As a result, E/A, E'/A' and E/E', all indicators of diastolic function, were not different between groups (Table 2). However, another index of diastolic function, isovolumetric relaxation time (IVRT), was slightly but significantly shorter in p/a-C1^{-/-} mice than in Wt mice. To determine whether the shortened IVRT in p/a-C1^{-/-} mice was an early sign of diastolic dysfunction, we repeated echocardiograms in a subgroup of mice when they were 7–8 month old. At this older age, none of the parameters studied differed between p/a-C1^{-/-} and Wt mice (data not shown), confirming preserved cardiac functioning in p/a-C1^{-/-} mice.

Overall the hearts of p/a-C1^{-/-} mice functioned normally and most if not all parameters were comparable between groups. However, because the heart has a remarkable capacity to maintain proper function and overcome minor defects, many cardiomyopathy phenotypes only become apparent when there is an increased demand on the heart. We therefore stressed the hearts of a subset of mice by injecting mice for 5 consecutive days with 20 µg/g of the β-adrenergic agonist, isoprenaline [29]. This treatment caused significant thickening of the left ventricular peripheral wall in both Wt and p/a-C1^{-/-} mice (Supplemental figure 4A) resulting in higher heart weight/body weight ratios (supplemental figure 4B) whereas control mice injected with saline showed no cardiac hypertrophy. However, isoprenaline treatment had no significant effects on cardiac function in either Wt or p/a-C1^{-/-} mice. None of the major parameters describing systolic (e.g. FS, SV) or diastolic (e.g. E, A, E' or IVRT) function changed upon treatment with isoprenaline. Cardiac function thus was not different in Wt and p/a-C1^{-/-} mice under these stress conditions (data not shown).

4. Discussion

In the study presented here we used a mouse model lacking the proline and alanine rich (p/a) region and the C1 domain of cMyBP-C [28] (p/a-C1^{-/-} mice) to investigate the *in vivo* relevance of these regions to sarcomeric and cardiac function. Unexpectedly, removal of these two regions of cMyBP-C did not cause overt contractile dysfunction, either at a cellular level or at the level of the whole heart of p/a-C1^{-/-} mice. This result is surprising considering the prevalence of multiple HCM polymorphisms in the C1 domain and the functional relevance of the p/a-and C1 regions demonstrated in a variety of *in vitro* assays [2, 24–26, 34].

cMyBP-C is thought to normally repress cross bridge cycling by hampering cross bridge formation or by forming a physical drag slowing down cross bridge kinetics. The C1 domain binds to myosin at the S2 region, near the hinge region at the junction of S1 and S2 [10, 35]. As a result, binding of cMyBP-C could restrain myosin heads and hamper cross bridge formation. Alternatively, the C1 domain could directly influence the position and so the function of the myosin heads [35]. The proline-alanine rich region and the C1 domain have both been implicated in mediating inhibitory or activating effects in *in vitro* assays, either by binding to myosin S2 or to actin [20–22, 36]. However, cardiomyocytes from p/a-C1^{-/-} mice not only had a similar maximal force development and Ca²⁺-sensitivity of tension as Wt controls, but the kinetics of force development were also comparable between the groups. These results suggest that the p/a-C1 region, at least in mice, is not essential for influencing cross bridge kinetics.

The finding that Ca^{2+} -sensitivity of tension was comparable in p/a-C1^{-/-} and Wt mice contrasts with increased Ca^{2+} -sensitivity of force originally reported by Witt *et al.* [28]. The different observations can potentially be explained by differences in treatment of the mice or myocardium and differences in experimental procedures, possibly leading to differences in protein status. However, because expression and phosphorylation levels of cMyBP-C and cTnI were not measured in the previous study a direct comparison of the results is difficult.

The p/a region and C1 domains further have been shown to be involved in the activating effect of the N-terminal domains (NTDs) of cMyBP-C in the absence of calcium, the regular activator of cross bridges [24, 25]. When bound to actin, the C0 and C1 domains of cMyBP-C bind in a position in which they clash with the “blocked” position of tropomyosin [37, 38], possibly biasing the thin filament into an active state. Shifting the position of tropomyosin towards its open configuration could thus be a mechanism by which cMyBP-C activates contraction in the absence of calcium. We hypothesized that such an activating mechanism could be important in sustaining contraction during late systole, when the cytosolic calcium concentration is declining. In support of a role for cMyBP-C in the time course of systole, Palmer *et al.* [19] reported that the absence of cMyBP-C in knockout mice accelerates the time course of systolic elastance, resulting in a shorter duration of systole. Our echocardiograms, however, showed no signs of systolic dysfunction in p/a-C1^{-/-} mice. The only parameter found to differ between p/a-C1^{-/-} and Wt mice was isovolumetric relaxation time (IVRT), which was slightly shorter in p/a-C1^{-/-} mice. A shorter IVRT in the absence of the p/a-C1 regions could indicate a change in diastolic function, but differences in IVRT were no longer significant in 7–8 month old mice suggesting no persistent diastolic phenotype in p/a-C1^{-/-} mice. None of the other functional parameters derived from the echocardiograms differed between p/a-C1^{-/-} and Wt mice and the hearts of p/a-C1^{-/-} mice appear to function normally despite lacking a substantial portion of cMyBP-C. Overall, our data suggest that the p/a-C1 region is not essential for the function of cMyBP-C at least not in mice.

Cross-species differences in the functions of specific regions of cMyBP-C might explain why mouse hearts function apparently well without the p/a-C1 region. Herron *et al.* identified the p/a region to have an activating effect on contraction using human peptides [24], whereas Razumova *et al.* found activating effects of cMyBP-C to be located in the C1 plus the M-domain of murine peptides [25]. Another study directly compared the activating effects of mouse and human cMyBP-C NTDs and confirmed that the human p/a region and C1 domain had greater activating effects on actomyosin interactions than murine p/a and C1 sequences [26]. Thus, it is likely that different regions are essential for the function of cMyBP-C in the mouse than those that are essential in human.

Alternatively, the regulatory function on cross bridge activation could be less pronounced in mammals with high heart rates such as mice because the duration of systole is shorter. If so, then the murine heart may be able to do well without the activating effects of the p/a and C1 regions. By contrast, *in vivo* data suggesting the relevance of the p/a region of cMyBP-C for cardiac function in humans came from a study by Kuster *et al.* [33], in which they identified that serine 133 in the p/a region can be phosphorylated by GSK3 β and that the maximal rate of force redevelopment increased in human cardiomyocytes upon incubation with GSK3 β .

Furthermore, the phosphorylation of serine 133 was found to be lower in samples of failing hearts compared to healthy hearts [33]. However, here we measured no difference in force production in skinned cardiomyocytes in either Wt or p/a-C1^{-/-} mice upon incubation with GSK3 β , again suggesting that the functional significance of the p/a region might differ between humans and mice.

The physiological relevance of the C1 domain in humans is not precisely known but its importance is suggested by the number of variants in this region found in hypertrophic cardiomyopathy patients [2, 34]. Whereas only one sequence variant linked to HCM was found in the p/a domain, a combination of 16 missense, nonsense and small deletions were found in the C1 domain [2, 34]. Ababou *et al.* showed that most mutations in the C1 domain are clustered towards the C-terminus of the domain and that these correlate with the binding site on myosin S2, suggesting that altered binding between C1 and myosin might contribute to causes of HCM [35].

It is still unclear what the role of individual domains of cMyBP-C is and how they interact with each other to exert their function. It is thus plausible that there is redundancy between certain domains of cMyBP-C that could explain the observed lack of effect of removal of the p/a and C1 domains in mice. For example, both the C0 and the C1 domains have been shown to bind actin [37, 39, 40]. Using electron microscopy and three-dimensional reconstruction, Orlova *et al.* demonstrated that C0C1 can bind to actin in two different conformations [40]. Although the resolution of the reconstructions was too low to discriminate which mass represents C0 or C1, these data suggest that there are multiple modes of interaction between cMyBP-C and actin. One could envision that binding of either domain to actin is sufficient for cMyBP-C to exert its function and that the two conformations thus are functionally equal. C1 has been shown to bind to myosin at the very beginning of S2, near S1-S2 hinge region and the light chains [35], while C0 binds nearby to the regulatory light chains [11]. This means that both the C0 and C1 domains may be located in close proximity to each other near the S1 heads and the myosin light chains, giving them the opportunity to influence the position of the S1 heads. It remains unresolved how modulation of S1 by cMyBP-C exactly influences cross bridge cycling, but it could be that C0 and C1 have similar effects on cross bridge cycling by their interactions with S1. Redundancy in domain function could also explain why absence of the C1 domain in the p/a-C1^{-/-} mice has no obvious effect, while sequence variants in the C1 domain are associated with HCM: in the absence of the C1 domain other domains might compensate for the function of C1, while a sequence variant in C1 could convert it into a 'poisonous peptide' leading to a dominant negative effect on the function of cMyBP-C. Additional high resolution structural and functional studies are needed to distinguish whether individual cMyBP-C NTDs are functionally similar or if they have unique effects on contraction.

In summary, our results indicate that the p/a and C1 region are not essential for function of the murine heart, despite indications that these regions are functionally relevant in human cMyBP-C and human cardiac function. This surprising finding highlights the need for a better understanding of the roles of individual domains of cMyBP-C and their influence on each other. Emerging insight in the structure of individual domains [35, 41, 42] and their binding partners [11, 40] will prove a valuable tool to identify the relevance of individual

domains of cMyBP-C. Additionally, more detailed insight in differences and similarities across species, together with insight in changes that occur in cMyBP-C within the wide spectrum of conditions under which the heart has to function, will be essential to a better understanding of how cMyBP-C tunes cardiac contraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cMyBP-C	cardiac myosin binding protein C
p/a region	proline-alanine rich region
p/a-C1^{-/-} mice	mice lacking the proline-alanine rich region and C1 domain of cMyBP-C
Wt mice	wild type mice
NTDs	N-terminal domains
CTDs	C-terminal domains
HCM	hypertrophic cardiomyopathy
cTnI	cardiac troponin I
cTnT	cardiac troponin T
PKA	protein kinase A
GSK3β	glycogen synthase kinase-3 beta

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Figure 1.

Schematic overview of the domains that make up cardiac myosin binding protein C (cMyBP-C). Ig-like domains are represented as ovals and FnIII-like domains as grey rectangles. The C0 and C1 domains are connected by proline-alanine rich region (p/a). A second linker region called the M-domain, or motif, is located between C1 and C2. The M-domain contains phosphorylation sites (asterisks) that modulate the interaction of cMyBP-C with other sarcomeric proteins. Regions of cMyBP-C that binds to other sarcomeric proteins are indicated. The region of interest studied in this work, the p/a region and C1 domain, is indicated by a green box.

Figure 2.

Cardiac myosin binding protein C (cMyBP-C) expression in hearts of p/a-C1^{-/-} and Wt mice. **A.** Immunostaining with antibodies directed against cMyBP-C (green) and α-actinin (red) demonstrated incorporation of cMyBP-C in doublets both in sarcomeres of Wt and p/a-C1^{-/-} mice. Image stacks were deconvolved using softWoRx® version 5.5 (GE Healthcare). Appropriate images were flattened into a single projection using Graphic Converter X version 6.7 (Lemke Software GmbH) and the “Merge folder into one Image” command. The images were histogram stretched and false colored in Photoshop CS5.1 (Adobe) with α-actinin in red and cMyBP-C in green. **B.** Coomassie stain of SDS-page gels or Western blots with antibodies raised against cMyBP-C of cardiac protein homogenates confirmed the presence of a shorter cMyBP-C in p/a-C1^{-/-} mice. **C.** Quantification of 1 of cMyBP-C expression demonstrated similar cMyBP-C expression in p/a-C1^{-/-} and Wt mice.

Figure 3.

A. Force-pCa relationships of p/a-C1^{-/-} and Wt mice before and after incubation with protein kinase A (PKA) demonstrated no differences in calcium sensitivity between groups. PKA incubation decreased calcium sensitivity in both groups as indicated by a rightward shift of the curve. **B.** Rate of force redevelopment at different pCa a **C.** levels of activation showed no significant differences between p/a-C1^{-/-} and Wt mice. *P<0.05 Wt versus p/a-C1^{-/-}.

Figure 4.

Phosphorylation of cMyBP-C (**A, D**), cardiac troponin I (cTnI) (**B, E**) and cardiac troponin T (cTnT) (**C, F**) was comparable between p/a-C1^{-/-} mice and Wt mice as determined by ProQ Diamond phosphoprotein stain. Treatment of the mice with propranolol for a week prior to their sacrifice lowered protein phosphorylation (Supplemental figure 1), but did not introduce differences between groups (**D, E, F**).

Figure 5. Representative echocardiograms from wild type (Wt) and $p/a-C1^{-/-}$ mice showing a B-mode short axis view (**A**), a parasternal long axis view (**B**), and an M-mode view showing traces used to analyze the morphometry and function of the hearts (blue lines) (**C**).

Table 1

Force development in cardiomyocytes from p/a-C1^{-/-} and wild type (Wt) mice.

	Wt (N=6)		p/a-C1 ^{-/-} (N=7)	
	- PKA	+ PKA	- PKA	+ PKA
F _{max} (kN/mm ²)	23.9±2.5	21.0±2.2	24.0±2.3	22.4±2.2
F _{min} (kN/mm ²)	3.4±0.7	2.8±0.4	2.9±0.2	2.4±.2
pCa ₅₀	5.93±0.04	5.70±0.02	5.90±0.02	5.72±0.02
k _{tr} (s ⁻¹)				
4.5	12.0±0.6	10.3±0.5	12.5±0.8	12.7±0.7
5.0	8.5±0.3	8.7±0.9	10.3±0.5	10.1±0.4
5.2	9.6±0.8	8.7±0.6	11.6±0.6	10.6±0.5
5.4	8.3±0.3	8.1±0.5	10.8±0.5*	10.5±0.8
5.6	8.1±0.7	6.4±0.5	9.1±0.3	8.1±0.5
5.8	5.8±0.7	3.7±0.7 ^[2]	7.2±0.4	4.1±0.3 ^[2]

Values are presented as mean ± SEM.

* P<0.05 Wt versus p/a-C1^{-/-}.

^[2] P<0.05 after versus before incubation with PKA.

Table 2

Summary of morphometric and functional characteristics of the hearts of p/a-C1^{-/-} and wild type (Wt) mice as assessed from transthoracic echocardiograms.

Measurement	Wt	n	p/a-C1 ^{-/-}	n
HR (bpm)	471±6	26	471±7	28
LVIDd (mm)	3.96±0.07	26	4.03±0.05	28
LVIDs (mm)	2.77±0.06	26	2.82±0.05	28
LVPWd (mm)	0.77±0.03	26	0.85±0.03	28
LVPWs (mm)	1.06±0.03	26	1.10±0.03	28
FS (%)	30.4±0.9	26	30.3±0.7	28
EF (%)	58.0±1.3	26	57.7±1.0	28
SV (ml)	39.9±1.4	26	41.3±1.3	28
CO (mL/min)	18.8±0.7	26	19.6±0.8	28
E (mm/s)	638.3±17.6	23	608.6±15.9	26
A (mm/s)	352.5±15.8	23	341.1±13.1	26
E/A	1.9±0.10	23	1.8±0.09	26
IVCT (ms)	13.6±0.55	23	14.1±0.54	26
IVRT (ms)	13.4±0.37	23	12.0±0.42	26
E' (mm/s)	-22.0±0.91	23	-20.6±1.17	24
A' (mm/s)	-18.9±0.78	23	-17.7±0.60	24
E'/A'	1.2±0.08	23	1.2±0.07	24
E/E'	-30.6±1.4	22	-31.9±2.1	24

Values are presented as mean ± SEM. HR, heart rate; LVIDd and LVIDs, left ventricular internal diameter diastolic and systolic; LVPWd and LVPWs, left ventricular posterior wall diameter diastolic and systolic; FS, fractional shortening; EF, ejection fraction; SV, stroke volume; CO, cardiac output; E and A, speed of blood flow over the mitral valve during early and late filling; IVCT and IVRT, isovolumetric contraction and relaxation time; E' and A', speed of tissue movement at the mitral valve annulus during early and late filling.