Molecular effects of the myosin activator omecamtiv mecarbil on contractile properties of skinned myocardium lacking cardiac myosin binding protein-C

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1. Introduction

Systolic dysfunction is a major cause of heart failure, and is characterized by reduced pumping ability of the heart and a significant reduction in the left ventricular (LV) ejection fraction [1]. The molecular basis of systolic heart failure is impaired myocyte contractile function at the sarcomere level, and specifically, the actomyosin cross-bridge (XB) cycle, which is the fundamental process that determines both the rate and magnitude of force generation in cardiac muscle [2]. Recent efforts to enhance myocardial contractility are directed toward targeting two of the elementary processes that occur during the XB cycle [2]. These efforts at the level of cardiac sarcomere involve: a) promoting the intrinsic rate of the transition of XB's from their weakly- to strongly-bound states, and b) reducing the amount of ADP release during the XB cycle in order to lower the energetic costs associated with the force-generating acto-myosin interactions. Thus, the cardiac sarcomere...

At the level of the sarcomere, one of the key proteins that regulate the dynamics of XB cycle is the cardiac myosin binding protein-C (cMyBP-C) [17–24]. Previously, studies have shown that skinned murine myocardium lacking cMyBP-C (KO) displayed significantly accelerated rates of XB detachment and recruitment leading to an overall acceleration in the XB cycling kinetics [25]. Accelerated XB cycling kinetics and mechanical dysfunction were also evident in the myocardium expressing even modest reductions in cMyBP-C expression (cMyBP-C+/−) [26,27]. Furthermore, accelerated XB kinetics have also been reported in skinned myocardium isolated from patients with similar deficits in cMyBP-C expression [28]. At the whole-heart level, the hypercontractile KO hearts displayed aberrant contractile efficiency as indicated by a reduced ejection fraction and an abbreviated ejection time [15,27, 29]. cMyBP-C+/− hearts displayed more modest systolic dysfunction and hypertrophy manifested as an elevated end-diastolic pressure and decreased peak rate of LV pressure rise [26,27].

We recently showed that in vivo reconstitution of cMyBP-C by gene transfer in KO hearts improved the in vivo systolic function and reduced cardiomyopathy [15]. Improved cardiac performance was primarily due to a normalization of XB behavior in the hypercontractile KO sarcomere due to increased cMyBP-C expression, which markedly slowed the rates of XB cycling [15]. Recently, omecamtiv mecarbil (OM), a cardiac myosin activator, has been shown to improve systolic function in the failing hearts [10,30], by enhancing XB-mediated force generation via enhancing the rate of transition of XB’s from the weakly-bound to the strongly-bound state [8], and decreases actomyosin in vitro motility velocity thereby increasing the overall XB duty cycle of the myosin motor [31]. Based on these findings, we investigated the utility of OM as a pharmacological approach to correct the molecular defects in the KO sarcomere which result in accelerated XB kinetics. Our findings indicate that incubation of skinned myocardium with OM significantly slowed XB kinetics in both wild-type (WT) and KO skinned myocardium. In particular, the acceleration of XB kinetics due to cMyBP-C ablation was largely blunted by OM incubation, suggesting that at the molecular level, OM may normalize the hypercontractile sarcomere.

2. Materials and methods

2.1. Ethical approval, animal incubation protocols, and procurement of donor human cardiac tissue samples

This study was performed as per the protocols given in the Guide for the Care and Use of Laboratory Animals and as per the guidelines of the Institutional Animal Care and Use Committee at Case Western Reserve University. Mice of either sex, aged 3–6 months (SV/129 strain), were used for the experiments. KO mice used in this study were previously generated and well-characterized [32]. WT mice expressing normal, full-length cMyBP-C (KO) displayed significantly accelerated rates of XB detachment and recruitment leading to an overall acceleration in the XB cycling kinetics [25]. Accelerated XB cycling kinetics and mechanical dysfunction were also evident in the myocardium expressing even modest reductions in cMyBP-C expression (cMyBP-C+/−) [26,27]. Furthermore, accelerated XB kinetics have also been reported in skinned myocardium isolated from patients with similar deficits in cMyBP-C expression [28]. At the whole-heart level, the hypercontractile KO hearts displayed aberrant contractile efficiency as indicated by a reduced ejection fraction and an abbreviated ejection time [15,27, 29]. cMyBP-C+/− hearts displayed more modest systolic dysfunction and hypertrophy manifested as an elevated end-diastolic pressure and decreased peak rate of LV pressure rise [26,27].

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2.2. Estimation of phosphorylation status of sarcomeric proteins in WT and KO heart samples

Cardiac myofibrils were isolated from frozen mouse ventricles [35]. In brief, a piece of the frozen tissue was thawed in a fresh relaxing solution, homogenized, and the myofibrils were then skinned for 15 minutes with 1% Triton X-100 [26]. Skinned myofibrils were then resuspended in fresh relaxing solution containing protease and phosphatase inhibitors (PhosSTOP and cComplete ULTRA Tablets; Roche Applied Science, Indianapolis, IN, USA) and stored on ice. To determine the myofilament protein phosphorylation status, ventricular samples were solubilized by adding Laemmli buffer and were heated to 90 °C for 5 minutes. For Western blot analysis, 2.5 μg of solubilized myofibrils were loaded onto a 4–20% Tris-glycine gel (Lonza Walkersville Inc., Rockland, ME, USA), transferred to PVDF membrane, and incubated overnight with one of the following primary antibodies: total troponin I (Tnl), Tnl phospho-serine 23 and 24 (detects phosphorylation of Ser23 and Ser24 of Tnl), total cMyBP-C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cMyBP-C phospho-serine 273, 282, or 302 (detects phosphorylation of cMyBP-C, Ser273, Ser282, or Ser302), or HSC70. For Pro-Q phosphoprotein analysis, 2.5 μg of solubilized cardiac myofibril samples were electrophoretically separated at 180 V for 85 minutes, then fixed and stained with Pro-Q diamond phosphoprotein stain (Invitrogen, Carlsbad, CA, USA) to assess the phosphorylation status of sarcomeric proteins. The same procedure was followed for testing the phosphorylation status in samples treated with OM. Densitometric scanning of the stained gels was performed using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA) [35].

2.3. Preparation of skinned ventricular myocardial preparations and Ca2+ solutions for experiments

Skinned ventricular myocardial preparations were prepared as described previously [26,35]. In brief, ventricular tissue was homogenized in a relaxing solution followed by detergent-skinning for 60 minutes using 1% Triton-X 100. Multicellular preparations measuring ~100 μm in width and 400 μm in length were selected for the experiments. The composition of various Ca2+ activation solutions used for the experiments was calculated using a computer program [36] and using the established stability constants [37]. All solutions contained the following (in mM): 100 N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg2+, and 4 MgATP. The maximal activating solution (pCa 4.5; pCa = −log [Ca2+]) also contained 7 EGTA and 7.01 CaCl2; while the relaxing solution (pCa 9.0) contained 7 EGTA and 0.02 CaCl2; and the pre-activating solution contained 0.07 EGTA. The pH of the Ca2+ solutions was set to 7.0 with KOH and the ionic strength of the Ca2+ solutions was 180 mM. A range of pCa solutions (pCa 6.6–5.5), containing varying amounts of [Ca2+]free, were then prepared by mixing appropriate volumes of pCa 9.0 and 4.5 stock solutions and all the experiments were carried out at 22 °C.

2.4. Experimental apparatus for the estimation of isometric force generation and force-pCa relationships in the skinned myocardium

Detergent-skinned ventricular preparations were held between a motor arm (312C, Aurora Scientific Inc., Aurora, Ontario, Canada) and a force transducer (403A; Aurora Scientific Inc.) as described previously [15,26]. Changes in the motor position and signals from the force transducer were sampled at 2.0 kHz using sarcomere length (SL) control software program [38]. For all mechanical measurements, SL of the ventricular preparations was set to 2.1 μm [26,27]. Force-pCa relationships were generated by incubating skinned myocardium in a range of pCa solutions (i.e., 6.6 to 4.5). The apparent cooperativity of force development was estimated from the steepness of a Hill plot transformation of the force-pCa relationships. The force-pCa data were fit using the
equation: \( P/P_0 = \left[ Ca^{2+}\right]^{dH}[(k_{M} + [Ca^{2+}]^{M})], \) where \( nH \) is the Hill coefficient and \( k \) is the pCa required to produce half-maximal activation (i.e., pCa50) [35].

2.5. Preparation of OM solution for incubating the myocardial preparations

OM was procured from Selleckchem (Houston, TX, USA) and was dissolved in DMSO (as per the manufacturer's instructions). OM stock solution was added to a relaxing solution to prepare a final concentration of 1 μM OM. The final concentration of DMSO in our solutions is 0.00625%, which has negligible effects on cardiac contractile function [39]. Basal contractile function was first measured in pCa solutions ranging from 6.6 to 4.5 following which the myocardial preparations were incubated for 2 minutes in a relaxing solution containing 1 μM OM. This incubation was followed by repeating the measurement of contractile function in the same myocardial preparation in pCa solutions ranging from 6.6 to 4.5. Measurements for force-pCa relationships were made at various levels of activator [Ca\(^{2+}\)] after a 2-minute incubation of the skinned myocardial preparations with OM.

2.6. Measurement of the rate of force redevelopment (k\(_d\))

k\(_d\) was measured in the myocardial preparations to assess XB transitions from both the weakly- to strongly-bound state and from the strongly- to weakly-bound states [40,41]. A mechanical slack-restretch protocol was used to measure k\(_d\) in the Ca\(^{2+}\)-activated myocardial preparations as described earlier [26,42,43]. Skinned muscle preparations were transferred from relaxing (pCa 9.0) to activating Ca\(^{2+}\) solutions (pCa ranging from 6.2 to 5.9), and once the myocardial preparations attained a steady-state isometric force, they were rapidly slackened by 20% of their original muscle length and were held for 10 ms. The slackening was followed by a brief period of unloaded shortening which causes a rapid decline in force because of the detachment of the strongly-bound XBs. The myocardial preparations were then rapidly restretched back to their original length and the time course of force redevelopment was measured. k\(_d\) was estimated by linear transformation of the half-time of force redevelopment, i.e., k\(_d\) = 693/t\(_{1/2}\), where t\(_{1/2}\) is the time (in milliseconds) taken to reach the half maximal force of the k\(_d\) trace as described previously [15,26,43–45]. Baseline force is considered the point on the k\(_d\) trace where force begins to redevelop following the slack-restretch maneuver, and peak force development is considered the point in the k\(_d\) trace in which force plateau and reaches a steady-state level.

2.7. Stretch activation experiments to measure the rates of XB detachment, XB recruitment, and XB stiffness

Stretch activation experiments were performed as described earlier [26,35,46,47]. Myocardial preparations were placed in Ca\(^{2+}\) solutions and were allowed to attain a steady-state force. Myocardial preparations were then rapidly stretched by 2% of their initial muscle length, held at the new length for 5 seconds and were then returned back to their initial muscle length. The characteristic features of the stretch activation responses in cardiac muscle have been described earlier [48,49], and the stretch activation parameters measured are shown in Fig. 4A. In brief, a sudden 2% stretch of muscle length causes an instantaneous rise in force (P1) produced by the myocardial preparation, which is due to the strain of elastic elements of the strongly-bound XBs (Phase 1). The force then quickly declines (Phase 2) due to a rapid detachment of the strained XBs which equilibrate into a non-force generating state, with a rate constant k\(_{ret}\). In Phase 2, XBs are both detaching and re-attaching with the rate of XB detachment greatly exceeding the rate of XB recruitment, and therefore k\(_{ret}\) measurement is an index of XB detachment. After this phase of quick force decline, the preparations exhibit a gradual force development (Phase 3), with a rate constant k\(_{det}\), due to stretch-induced recruitment of new XBs into the force-generating state [35,49].

In phase 3, XBs are both detaching and re-attaching with the rate of XB recruitment greatly exceeding the rate of XB detachment, and therefore k\(_{det}\) measurement is an index of XB recruitment. Stretch activation amplitudes were normalized to pre-stretch Ca\(^{2+}\)-activated force and were measured as described previously [27,35]. k\(_{ret}\) and k\(_{det}\) were estimated using a linear transformation of the force decay and force redevelopment, respectively. k\(_{ret}\) was measured by fitting a single exponential to the time course of force decay (using the formula: \( y = a \times (1 - \exp(-k_r \times x)) \) where a is the amplitude of the single exponential phase and \( k_r \) is the rate constant of the force decay as described previously [25,50,51].

k\(_{det}\) was measured by linear transformation of the half-time for force redevelopment using the formula: \( k_{det} = -\ln(0.5) \times (t_{1/2})^{-1} \) where \( t_{1/2} \) is the time (in milliseconds) taken from the nadir to half the maximal force in phase 3 of the force response shown in Fig. 4A, where maximal force is indicated by a plateau region in phase 3 as described previously [25,26,50,51].

2.8. Data analysis

All data are reported as mean ± SEM. One-way analysis of variance (ANOVA) was used to test whether there are any significant differences in the mean values from multiple groups [52]. Independent t-tests were used to assess whether there are any significant differences between two different groups, and paired t-tests were used to assess whether there are any significant differences pre- and post-OM treatment within the same group [35]. Correlation analysis was performed to substantiate the trends in pCa vs. % decreases in the force decay and force redevelopment, respectively. k\(_{ret}\) was calculated by linear transformation of the half-time for force redevelopment using the formula: \( k_{ret} = -\ln(0.5) \times (t_{1/2})^{-1} \) where \( t_{1/2} \) is the time (in milliseconds) taken from the nadir to half the maximal force in phase 3. Significant differences were set at \( P < 0.05 \) and the asterisks in figures and tables represent statistical significance using t-tests.

3. Results

3.1. Effect of OM on the phosphorylation levels of sarcomeric proteins

To determine if OM treatment alters the phosphorylation status of key regulatory sarcomeric proteins, WT and KO myocardial samples were subjected to Western blot and Pro-Q phospho-analysis prior to and following treatment with OM (Fig. 1). Our Western blot analysis shows that in WT samples, phosphorylation of TnI at residues Ser23/24 and cMyBP-C at Ser273, Ser282, and Ser302 was unaltered by OM treatment (Fig. 1A). TnI phosphorylation was similarly unaltered by OM treatment in KO samples and cMyBP-C phosphorylation and its total protein was not detected in KO samples (Fig. 1A). Ventricular samples from WT and KO hearts were also stained with Pro-Q Diamond stain to assess the effects of OM treatment on the phosphorylation levels of various regulatory myofilament proteins (Fig. 1B). As we previously reported [53] the phosphorylation levels of various sarcomeric proteins such as cardiac TnT, cardiac TnI and regulatory light chain were not significantly different between WT and KO myocardial samples (Fig. 1B). Furthermore, our data also shows that treatment with OM did not affect the phosphorylation status of myofilament proteins in both WT and KO hearts as shown by the absence of significant differences between pre and post OM samples within WT and KO groups (Fig. 1C).

3.2. Effect of OM on Ca\(^{2+}\)-activated force generation in WT and KO myocardial preparations

Ca\(^{2+}\)-activated force production was first measured in skinned ventricular preparations in Ca\(^{2+}\) solutions with increasing amounts of Ca\(^{2+}\) (pCa 6.2, 6.1, 6.0, and 5.9, i.e., –10 to 40% of maximal force [53]). This was followed by a 2-minute incubation of the preparations in 1.0 μM OM and measuring the Ca\(^{2+}\)-activated force production in pCa solutions: 6.2, 6.1, 6.0, and 5.9. Our results indicate that there was a significant increase in the force production in both WT and KO preparations after 2-minute incubation with OM (Fig. 2). Furthermore, our results

\[ \text{equation: } P/P_0 = \left[ Ca^{2+}\right]^{dH}/(k_{M} + [Ca^{2+}]^{M}) \]
show that the % increase in force from baseline (pre-OM) to post-OM was more pronounced at low levels of activator \([\text{Ca}^{2+}]\) (pca 6.2) and progressively decreased as the level of activator \([\text{Ca}^{2+}]\) increased (Fig. 2) in skinned mouse myocardium. Similar trends were observed in preparations isolated from human left ventricular samples which predominantly express the slow/β-MHC isoform, although, the force enhancement was less pronounced than what was observed in the predominantly α-MHC background of the mouse myocardium (WT and KO). For human heart preparations, the % increases in force production from the baseline were 60.4 ± 14.6, 49.5 ± 10.8, 27.6 ± 9.9, and 14.0 ± 4.8, respectively at pca’s 6.2, 6.1, 6.0, and 5.9. Furthermore, incubation with OM did not affect the force generation at maximal Ca\(^{2+}\) activation (pCa 4.5) in any of the groups (Table 1).

3.3. Effect of OM on myofilament Ca\(^{2+}\) sensitivity (pCa\(_{50}\)) and cooperativity of force development (n_H)

The effect of OM on pCa\(_{50}\) was assessed by plotting normalized force values against a range of pCa and constructing force-pCa relationships at SL 2.1 μm in WT, KO, and human heart preparations. pCa\(_{50}\), the pCa required to generate half-maximal force, was estimated by fitting the Hill equation to the force-pCa relationships. Our data shows that treatment with OM increased the responsiveness of the cardiac myofilaments to Ca\(^{2+}\) at submaximal Ca\(^{2+}\)–activations as indicated by a significant left-ward shift in the force-pCa relationships in all the groups (Figs. 3A, B, and C). pCa\(_{50}\) values for WT, KO, and human heart preparations are shown in Table 1. The effect of OM on n_H was assessed by fitting the Hill equation to the force-pCa relationships. n_H values decreased post-OM treatment in all the groups indicating that incubation with OM decreased the overall cooperativity of force production (Table 1).

3.4. Effect of OM on the rate of force redevelopment (k_tr) in WT and KO myocardial preparations

k_tr is an index of XB transition from both the weakly- to strongly-bound state and from the strongly- to weakly-bound states [40,41] and OM has been shown to accelerate the transition of XBs to the

Fig. 1. Western Blot and Pro-Q analysis to assess the phosphorylation status of myofilament filament proteins in WT and KO heart preparations. (A) Western blots showing cMyBP-C and Tnl phosphorylation before and after treatment with OM in WT and KO heart samples. Tnl phosphorylation at residues Ser23/24 was similar between WT and KO samples and was unaffected by OM treatment. cMyBP-C phosphorylation of residues Ser273, Ser282, and Ser302 was absent in KO tissue, and their phosphorylation levels in WT was unaffected by OM treatment. (B) Representative Pro-Q Diamond-stained (left) and Coomassie stained (right) SDS gel showing the phosphorylation status of myofilament proteins before and after treatment with OM in WT and KO heart samples. (C) Quantification of protein phosphorylation as determined by Pro-Q and Coomassie stains from 6 WT and 6 KO hearts. The intensity of the phosphorylation signal was normalized to the intensity of the total protein signal and the untreated WT myofilament protein phosphorylation was set to 1 as done in our previous study [35]. cMyBP-C phosphorylation was unaffected by OM treatment in WT preparations and was absent in KO preparations. No differences in phosphorylation status of myofilament proteins were observed between WT and KO hearts. Furthermore, treatment with OM for 2 minutes did not induce any significant changes in the phosphorylation status of myofilament proteins in WT and KO hearts. WT, wild-type; KO, knockout; cMyBP-C, cardiac myosin binding protein-C; cTnT, cardiac troponin T; cTnI, cardiac troponin I; RLC, regulatory light chain.

Fig. 2. Effect of OM on force enhancement at various levels of activator \([\text{Ca}^{2+}]\). Baseline forces generated by the skinned ventricular preparations were first measured in Ca\(^{2+}\) solutions with pCa ranging from 6.2 to 5.9. Forces were again measured on the same preparations using the same range of pCa solutions following 2-minute incubation with OM. The net increase in force generation following incubation with OM at each level of activator \([\text{Ca}^{2+}]\) was calculated and is expressed as % increase in force from baseline in (A) WT and (B) KO preparations. Thus, the % increases shown in panels A and B following OM treatment are over and above the Ca\(^{2+}\)–mediated force generation, at each pCa. The ability of OM to enhance force production decreases as the level of activator \([\text{Ca}^{2+}]\) in the sarcomere increases. No statistical differences were found between the % increases in forces following OM treatment in WT and KO groups. This indicates that OM enhanced force generation in the WT and KO preparations to the same extent. Values are expressed as mean ± S.E.M. Independent t-tests were used to compare the data between WT and KO groups and paired t-tests were used to compare the data between pre- and post-OM treatment with in the same group. 16 preparations were analyzed from 5 WT hearts and 18 preparations were analyzed from 5 KO hearts, with multiple preparations from each heart. * P < 0.05 when comparing forces generated before incubation with OM vs. forces generated following incubation with OM within each group.

A

B

C

D
strongly-bound force-producing state in an in vitro assay [8]. In this study we wanted to test the effects of OM on $k_{tr}$ under conditions where the cardiac sarcomeric lattice structure is intact. To gain better insights regarding the effect of OM on $k_{tr}$, we measured $k_{tr}$ at the same level of activator [Ca$^{2+}$] (pCa 6.1) before and following incubation with OM. The force produced was significantly higher at the same level of activation (i.e., force generation, [41,43]). Therefore, because OM incubation produced a dramatic increase in force generation at pH 6.1 in both WT and KO preparations (Fig. 2), one would also expect to observe parallel and significant increases in $k_{tr}$. However, no such increases in $k_{tr}$ were observed at pH 6.1 (Fig. 5A) even when there were significant increases in force production (Fig. 2) following OM incubation. At slightly higher pH (pCa = 6.0), it is clear that OM treatment significantly decreased $k_{tr}$ (Fig. 5B). Thus, our data indicate that the rate of transition of XBs into the force-bearing state is decreased following incubation with OM.

3.5. Effects of OM on the rates of XB detachment ($k_{rel}$) and XB recruitment ($k_{rec}$) in WT and KO myocardial preparations

Our data show that OM slows the XB turnover rate, $k_{rel}$ in WT and KO preparations (Fig. 5; Table 2). Because $k_{rel}$ is proportional to the sum of $f$ (rate of XB recruitment) + $g$ (rate of XB detachment) according to a simple two-state XB model [40], we tested whether the OM-induced decrease in $k_{rel}$ arises from changes in either the rate of XB detachment or the rate of XB recruitment, or due to both. We used stretch activation experiments (see the Methods section) to measure $k_{rel}$ and $k_{rec}$ which

<table>
<thead>
<tr>
<th>Group</th>
<th>pCa$_{50}$ (nm)</th>
<th>$n_H$ (unitless)</th>
<th>$F_{max}$ (mN/mm$^2$)</th>
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<tbody>
<tr>
<td>Pre-OM</td>
<td>WT</td>
<td>5.83 ± 0.02</td>
<td>2.42 ± 0.13</td>
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<tr>
<td></td>
<td>KO</td>
<td>5.86 ± 0.01</td>
<td>2.33 ± 0.14</td>
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<tr>
<td></td>
<td>Human</td>
<td>5.85 ± 0.02</td>
<td>2.49 ± 0.12</td>
</tr>
<tr>
<td>Post-OM</td>
<td>WT</td>
<td>5.97 ± 0.03*</td>
<td>1.92 ± 0.17*</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>6.01 ± 0.02*</td>
<td>1.87 ± 0.08*</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>5.92 ± 0.02*</td>
<td>2.07 ± 0.12*</td>
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pCa$_{50}$: myofilament Ca$^{2+}$ sensitivity; $n_H$: cooperativity of force production; $F_{max}$: force generation at maximal Ca$^{2+}$ activation (pCa 4.5). Independent t-tests were used to compare the pre-OM and post-OM data in WT, KO, and human groups. 14-20 preparations were analyzed from 3 human hearts with multiple preparations from each heart. Values are expressed as mean ± S.E.M. * Significantly different from the corresponding pre-OM group; P < 0.05.
are indices of the rates of XB detachment and XB recruitment, respectively [26,35].

When studied at the same level of activator [Ca\(^{2+}\)] (pCa 6.1), we found that \(k_{\text{rel}}\) was significantly slowed following incubation with OM in both WT and KO preparations (Fig. 6A). As shown previously [15, 50,53] KO preparations exhibited an increased basal \(k_{\text{rel}}\) when compared to WT preparations. However, following incubation with OM, the decrease in \(k_{\text{rel}}\) was more pronounced (at pCa 6.1) in the KO preparations (~76%) when compared to the WT preparations (~58%), such that the differences observed in baseline \(k_{\text{rel}}\) between WT and KO preparations were no longer apparent (Table 5 and Fig. 6A). A similar trend was observed in the human skin myocardium as \(k_{\text{rel}}\) decreased by 32% post OM incubation (Table 3).

Akin to our observations at the same level of activator [Ca\(^{2+}\)], \(k_{\text{rel}}\) was significantly decreased following incubation with OM in WT and KO preparations when force-matched comparisons were made (Table 2). The differences observed in \(k_{\text{rel}}\) between WT and KO groups before incubation with OM were no longer apparent following incubation with OM (Table 2). Such significant decreases in \(k_{\text{rel}}\) following incubation with OM were also seen in the control human heart preparations (Table 4). Furthermore, the decrease in \(k_{\text{rel}}\) following incubation with OM was progressively diminished as the level of activator [Ca\(^{2+}\)] increased in both WT and KO groups (Table 5). Similar trends were observed in the human heart preparations. The percentage decreases in \(k_{\text{rel}}\) were 39.30 ± 7.33, 41.92 ± 10.20, 35.37 ± 6.37, 17.11 ± 2.62, respectively at pCa’s 6.2, 6.1, 6.0 and 5.9 in human heart preparations. Collectively, our results suggest that incubation with OM slows the overall XB cycling kinetics and prolongs the duty cycle of the myosin heads. In particular, slowed XB detachment rate (\(k_{\text{rel}}\)) would act to maintain thin filament activation for a longer time period which can then increase the force production (Fig. 2) by allowing enhanced OM-mediated cooperative XB recruitment and binding to open actin molecules [55], which contributes to an overall decrease in \(k_{\text{rel}}\) and \(k_{\text{eff}}\) [41]. Thus, slowed \(k_{\text{rel}}\) is likely a secondary effect of the slowed XB detachment-induced prolongation in the XB duty cycle.

### 3.6. Effect of OM on XB stiffness (P1) in WT and KO myocardial preparations

Using stretch activation experiments we tested whether incubation with OM affects the XB stiffness. We imposed a sudden 2% stretch in muscle length in an isometrically-contracting myocardial preparation and measured the magnitude of the elicited instantaneous increase in force (P1 in Fig. 4A). P1 is a result of a rapid distortion of the elastic regions of the strongly-bound XB5s and is an index of the XB stiffness [26, 48,56]. As shown previously [53] our current data shows that KO preparations exhibited reduced XB stiffness when compared to the WT preparations (Table 2). When the level of Ca\(^{2+}\)-activation was equivalent, P1 following OM incubation displayed a trend towards increased P1 in both WT and KO preparations, and when pre-stretch force was matched, XB stiffness was significantly increased in both WT and KO skinned preparations as indicated by increases in P1 (Table 2). At equivalent levels of activator Ca\(^{2+}\) (pCa 6.1), P1 was not affected following OM incubation in human myocardial preparations (Table 3), and P1 increased significantly in human myocardial preparations when the pre-stretch forces levels were matched (Table 4).

### 4. Discussion

In this study we tested the utility of OM in attenuating the accelerated contractile XB kinetics associated with the absence of cMyBP-C in the cardiac sarcomere. Our data shows that both the rates of XB detachment and...
from the strongly-bound state and XB recruitment into the force-generating state are significantly slowed in the KO skinned myocardium following incubation with OM, suggesting that pharmacological intervention can be considered to slow hypercontractile XB kinetics in myocardium expressing reduced levels of cMyBP-C.

### 4.1. The effect of OM on steady-state force generation depends on the level of Ca\(^{2+}\) activation

It is known that OM increases myocardial contractility by directly activating the cardiac myosin motor, unlike other commonly clinically used inotropic drugs such as \(\beta\)-adrenergic receptor agonists and phosphodiesterase inhibitors which activate the signaling pathways that ultimately enhance the intracellular Ca\(^{2+}\) transients [57]. In particular, OM infusion has been shown to increase LV systolic ejection time in the dog model of systolic heart failure [30] and has been shown to increase cardiac contractility as indicated by significant increases in fractional shortening in rat and dog models [8]. However, to date, the effects of OM on cardiac contractility in the presence of varying levels of activator [Ca\(^{2+}\)] in skinned myocardium has not been studied. Here we found that incubation of skinned myocardium with OM significantly increased the force generation at low levels of activator [Ca\(^{2+}\)] and significantly increased the myofilament Ca\(^{2+}\) sensitivity (Fig. 2) and indicating that more XBs are being recruited into the force-producing state following incubation with OM (Fig. 2), however, the magnitude of force enhancement was inversely proportional to the levels of activator [Ca\(^{2+}\)], and progressively decreased with increasing [Ca\(^{2+}\)] (Fig. 2). Our data also shows that the increase in myofilament Ca\(^{2+}\) sensitivity (\(p_{\text{Ca}}\)) following incubation with OM was also accompanied by an overall decrease in the Hill coefficient of force production (i.e., shallower \(n_{\text{H}}\)) (Table 1) indicating enhanced cooperative XB recruitment. Post-OM incubation, increases in force production were more pronounced at low levels of activator Ca\(^{2+}\) and progressively decreased with increased levels of activator Ca\(^{2+}\) (Fig. 2). This phenomenon is likely due to the fact that at low Ca\(^{2+}\) activations thin filament activation is more reliant on XB-mediated cooperative XB recruitment because most of the thin filament regulatory units (RU) are in their off state [55]. At high [Ca\(^{2+}\)] most of the RU’s will already be in their on state, which reduces the reliance of the thin filament on XB-mediated cooperative XB recruitment. Because OM promotes enhanced force generation through increased cooperative XB recruitment, the effects on force generation are more pronounced at low Ca\(^{2+}\)-activations, thereby resulting in a decrease in the steepness of \(n_{\text{H}}\) (Table 1) [58]. Similar trends were observed in donor human heart preparations but the magnitudes of the force enhancement at various levels of activator [Ca\(^{2+}\)] were less pronounced following OM incubation.

Differences in the magnitude of OM-induced force enhancements between murine and human heart preparations may be related to the inherent kinetic differences in the isofrom expression of MHC. Human hearts predominantly express the \(\beta\)-MHC isofrom which is an inherently slow myosin motor with a longer XB duty ratio [59–62] than the fast \(\alpha\)-MHC isofrom motor expressed in the mouse heart. Consequently, it is possible that the apparent prolongation of the duty ratio induced by OM may be less prominent in the already slow \(\beta\)-MHC-XBs in the human myocardium compared to the fast \(\alpha\)-MHC-XBs in the murine myocardium, thereby, explaining the relatively smaller effects of OM-induced force enhancements observed in human myocardium at various [Ca\(^{2+}\)]. Regardless, the effects of OM on force enhancement at low levels of activator [Ca\(^{2+}\)] were still highly significant in the human myocardium (Table 3). A greater effect of OM on force generation at lower levels of activator [Ca\(^{2+}\)] is consistent with a mechanism that involves cooperative XB-recruitment, which is more pronounced at low levels of Ca\(^{2+}\)-activation [41,54] where relatively few XBs are in the strongly-bound force-producing states and the recruitment of additional XBs to strongly bound-states can increase force generation significantly. Furthermore, positive feedback effects of the additionally recruited XBs will further help to stabilize the open conformation of the N-terminus of cardiac TnC to sustain the thin filament activation and enhance force generation at low Ca\(^{2+}\) levels [63,64].

### 4.2. OM-induced slowing of XB kinetics are more pronounced in the hypercontractile KO skinned myocardium

It has been shown that OM allosterically activates the myosin motor by binding to the catalytic domain of the myosin head and promotes the transition of XBs into a strongly-bound, force-generating state [8], and has also been shown to induce a large increase in the average duty cycle of porcine ventricular myosin [31]. Considering that KO skinned myocardium displays a significant acceleration in XB cycling kinetics, OM’s ability to increase the duty cycle of the myosin motor may be a useful pharmacological approach to correct contractile dysfunction in the KO myocardium. Because \(k_{\text{fi}}\) is an index of rate of transition of XBs from the weakly- to strongly-bound, and strongly- to weakly-bound states [40,41], we used a slack-restretch maneuver [35,53] to directly measure the impact of OM on \(k_{\text{fi}}\). When studied at the same level of activator [Ca\(^{2+}\)], we found that \(k_{\text{fi}}\) was unaffected following incubation.
with OM in both WT and KO preparations (Fig. 5A), and when steady-state forces were matched, $k_{tr}$ was significantly decreased following OM incubation (Fig. 5B, Table 2). This result was somewhat surprising because we have previously shown that $k_{tr}$ increases significantly in both WT and KO preparations as the amount of force generation increases [43]. Our finding that $k_{tr}$ was blunted, or even decreased, despite a large increase in the steady-state force generation following incubation with OM in both WT and KO preparations (Fig. 2), suggests that OM inherently decreases $k_{tr}$. Decreased $k_{tr}$ was also observed following incubation with OM in the human skinned myocardium.
both at matched Ca^{2+}-activation levels (Table 3) and at matched force generation levels (Table 4), indicating that qualitatively, the effects of OM on $k_{rel}$ were not dependent on the MHC isoform that is expressed in cardiac muscle. Decreased $k_{rel}$ following OM incubation could be due to decreased rates of transition of XBs into the force-bearing state or due to decreased rates of XB detachment, or both.

Therefore, we performed stretch-activation experiments to further probe the effects of OM on the rates of XB detachment ($k_{rel}$) and the XB recruitment ($k_{rec}$). Our stretch-activation data reveal that both $k_{rel}$ and $k_{rec}$ decreased significantly post-OM incubation, whether these rates were measured at same level of activator [Ca^{2+}], or at equivalent levels of pre-stretch force generation (Fig. 6; Table 2). In particular, differences in $k_{rel}$ that were observed between WT and KO prior to OM incubation (i.e., KO exhibited significantly accelerated $k_{rel}$ at baseline) were abolished following OM incubation (Table 2) indicating the rates of XB detachment in KO preparations were decreased to a greater extent than in the WT preparations, specifically at pCa’s 6.1 to 5.9 (Table 5). Furthermore, the effects of OM on $k_{rel}$ were more pronounced in KO skinned myocardium, in that $k_{rel}$ was significantly decreased at all levels of activator [Ca^{2+}] studied, whereas the decrease in $k_{rel}$ in WT skinned myocardium progressively reduced as the levels of activator [Ca^{2+}] increased (Table 5). Reduced XB detachment rates (i.e., $k_{rel}$ following OM incubation also predicts that ATP consumption during the XB bridge cycle (i.e., tension cost) is significantly reduced because XB detachment rate is highly correlated with tension cost [40,65–67]. The decrease in $k_{rel}$ may also be related to increased XB stiffness that was observed in WT and KO preparations following OM incubation [53] (Table 2). The increased XB stiffness along with the related conformation changes in the myosin heads may have likely increased the recruitment time of the XBs to actin, maintaining the XBs for a longer time period in their force-generating state by resisting strain-induced XB detachment (Fig. 6). Taken together, these effects would be predicted to enhance force generation and concomitantly increasing the efficiency of muscle contraction, as has been shown in previous studies [30]. On the other hand, it may seem counter-intuitive that increased force generation following OM incubation (Fig. 2) resulted in a decreased $k_{rel}$ (Fig. 6). However, decreased $k_{rel}$ may be due to the fact that enhanced XB recruitment involves both Ca^{2+} and XB-mediated cooperative spread of thin filament activation that aids in the transition of additional XBs from the non-cycling pool or a weakly-bound state to the strongly-bound state. Because cooperative activation is a time consuming process it acts to limit the overall rate of force development [41]. Therefore, it is likely that OM primarily acts as a XB-mediated activator of the thin filament which sustains the already bound XBs in their attached state for a longer period. The potential secondary effect of prolonged XB recruitment time is that thin filament regulatory units remain open for a longer time period, thereby enhancing the access of unbound XBs to bind to open actin sites. In this regard, consistent with $k_{rec}$ findings, a significant decrease in both $k_{rel}$ and $k_{rel}$ post-OM incubation was also observed in the human preparations that predominantly express β-MHC isoform (Tables 3 and 4), further confirming that the effects of OM on XB behavior are not cardiac MHC isoform dependent.

4.3. Potential in vivo consequences of OM-induced slowing of XB kinetics in the KO myocardium

Deficiency of cMyBP-C accelerates XB cycling kinetics and reduces the time that XBs effectively spend in their force-generating state, thereby, contributing to a premature truncation of the systolic ejection phase in vivo [68,69]. In this study, we observed a large increase in the force generation at low levels of activator [Ca^{2+}] following OM incubation (Fig. 2) which is due to decreased rates of XB detachment which prolongs XB cycle dynamics and enhances cooperative XB-mediated XB recruitment. In this regard, a recent report [31] demonstrated a 10-fold increase in the duty cycle of OM-treated isolated pig cardiac myosin, which would be predicted to significantly enhance force generation. These molecular effects of OM on XB behavior can be predicted to significantly promote the recruitment of XBs into the force-bearing state at the initial phase of isovolumic contraction in vivo when [Ca^{2+}] levels in the sarcomere are still relatively low. It is also possible that the OM-induced slowing of XB detachment may subsequently prolong duty cycle of the force-bearing state of XBs later in systole even at a time point when the cytosolic Ca^{2+} levels wane off, specifically, extending the period of the late systolic ejection phase, as shown previously [8,30]. On the other hand, although, prolonged systolic ejection due to delayed XB detachment could significantly augment systolic function and cardiac output, such a mechanism could also have an adverse impact on diastolic function if systole is sufficiently prolonged such that it impinges on the period of diastolic relaxation which would delay diastolic filling, a possibility that requires further investigation.

4.4. Limitations of the study

Our experiments at the myofilament level shows that treatment with OM can be a potential pharmacological approach to slow XB kinetics in myocardium lacking cMyBP-C, but extrapolating these findings to
the whole organ level is premature. The present study was performed in isolated skinned myocardial preparations where the level of Ca\(^{2+}\) activation and experimental conditions are precisely controlled. However, modulation of cardiac function at the whole organ level is much more complex, involving a complex interplay of the myofilaments with the cellular Ca\(^{2+}\)-handling machinery, neurohormonal signalling, electrical activation, etc. Thus, it is also possible that the molecular findings presented here may not completely translate to benefits at the whole heart level in the KO model. Therefore, future experiments will have to be performed to investigate the utility of OM in improving in vivo contractile and hemodynamic function in cMyBP-C deficient hearts under basal conditions, and also in response to increased workload, such as with increased β-adrenergic stimulation. These in vivo experiments will provide vital insights into the effectiveness of OM infusion in improving cMyBP-C-related contractile dysfunction.

4.5. Conclusions

Altered XB contractile dynamics is a common feature of animal models and human patients with hypertrophic cardiomyopathy (HCM). Increased k\(_{\text{p}}\) and rates of XB detachment have been observed in skinned myocardium isolated from patients expressing HCM-causing mutations in cMyBP-C and MHC [28,70,71], and are thought to contribute to increased tension cost of contraction [72]. In this study, we show a significant slowing in the intrinsically faster XB kinetics in the skinned KO myocardium following OM incubation. Because hypercontractile XB behavior is thought to be an important feature in the pathogenesis of cMyBP-C and MHC-related HCM, the pharmacological use of OM may have utility in normalizing contractile function at the level of sarcomere, and therefore, the effects of OM on improving in vivo function should be investigated.

Conflict of interest statement

There are no conflicts of interest.

Author and contributions

R.M. K.S.G, and J.E.S contributed to the conception and design of the experiments. R.M., K.S.G A.L., C.G.D.R., and J.E.S participated in performing the experiments, data acquisition, data analysis, data interpretation, drafting, and revising the manuscript. All authors approved the final version of the manuscript.

Acknowledgements

This work was supported by the National Heart, Lung, and Blood Institute Grant (HL-114770).

References


