Omecamtiv Mecarbil Enhances the Duty Ratio of Human β-Cardiac Myosin Resulting in Increased Calcium Sensitivity and Slowed Force Development in Cardiac Muscle

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The small molecule drug omecamtiv mecarbil (OM) specifically targets cardiac muscle myosin and is known to enhance cardiac muscle performance, yet its impact on human cardiac myosin motor function is unclear. We expressed and purified human β-cardiac myosin subfragment 1 (M2β-S1) containing a C-terminal Avi tag. We demonstrate that the maximum actin-activated ATPase activity of M2β-S1 is slowed more than 4-fold in the presence of OM, whereas the actin concentration required for half-maximal ATPase was reduced dramatically (30-fold). We find OM does not change the overall actin affinity. Transient kinetic experiments suggest there are two kinetic pathways in the presence of OM. The dominant pathway results in a slow transition between ADP states and increases the time myosin is strongly bound to actin. However, OM also traps a population of myosin heads in a weak actin affinity state with slow product release. We demonstrate that OM can reduce the actin sliding velocity more than 100-fold in the in vitro motility assay. The ionic strength dependence of in vitro motility suggests the inhibition may be at least partially due to drag forces from weakly attached myosin heads. OM causes an increase in duty ratio examined in the motility assay. Experiments with permeabilized human myocardium demonstrate that OM increases calcium sensitivity and slows force development (kDuty) in a concentration-dependent manner, whereas the maximally activated force is unchanged. We propose that OM increases the myosin duty ratio, which results in enhanced calcium sensitivity but slower force development in human myocardium.

Heart failure continues to be a major health problem worldwide, and despite current treatment options, the 5-year mortality rate remains relatively high (42%) (1, 2). In systolic heart failure, inotropic drugs, including β-agonists and phosphodiesterase inhibitors, are utilized to enhance cardiac muscle contractile force in individuals that are hypocontractile (3). However, prolonged use of these drugs is associated with increased myocardial oxygen demand, arrhythmias, and impaired calcium signaling (4, 5). Recently, drugs are being pursued that directly interact with cardiac myosin, the molecular motor that drives contraction in the heart, to enhance contractile force without altering intracellular calcium concentrations (6).

Muscle contraction is driven by an ATP-dependent cyclic interaction between the myosin thick filaments and actin thin filaments, with contraction and relaxation dependent on calcium-mediated changes in the thin filament regulatory proteins troponin/tropomyosin (7). Thick filament-associated proteins also play a role in regulating contraction (8, 9). A solid understanding of the conserved actomyosin ATPase pathway has emerged from decades of biochemical, biophysical, and structural studies (Scheme 1) (10, 11). ATP binding to actomyosin (K', K') dramatically weakens the affinity of myosin for actin and rapidly dissociates the complex, which allows for ATP hydrolysis to occur in a detached state (K'). Thus, the M-ATP and M-ADP-Pi states are referred to as weak actin binding states (boldface in Scheme 1), and the M-ADP and nucleotide-free states are referred to as strong actin binding states. The binding of M-ADP-Pi to actin triggers the myosin power stroke that is followed by an essentially irreversible phosphate release step (K'P) (12, 13). A second power stroke is thought to occur after phosphate release but is controversial (before K'Sb) (12, 14). The release of ADP from the active site (K'Sb) is the step that limits detachment from actin and is thus thought to be the step that limits maximum shortening velocity in muscle (15). However, attachment-limited models have also been proposed that can explain maximum shortening velocity in the context of the myosin ATPase cycle (16, 17).

Omecamtiv mecarbil (OM)2 is an allosteric modulator of human β-cardiac myosin that is currently in phase II clinical trials (18–20). OM was found to be specific for cardiac myosin.

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2The abbreviations used are: OM, omecamtiv mecarbil; mant, N-methylanthraniloyl; ELC, essential light chain.

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This article contains supplemental Fig. S1.
Impact of Omecamtiv Mecarbil on Cardiac Myosin

**Scheme 1**

\[\begin{align*}
K_1 & \quad K_2 \\
M + ATP & \leftrightarrow M_{ATP} \leftrightarrow M_{ADP, Pi} \\
K_3 & \quad K_4 \\
AM + ATP & \leftrightarrow AM_{ATP} \leftrightarrow AM_{ADP, Pi} \\
K_5^A & \quad K_5^B \\
AM^*_{ADP} & \leftrightarrow AM_{ADP} \leftrightarrow AM \\
\end{align*}\]

**Results**

**Purification of M2β-S1**—We obtained \(~0.75–1.0\) mg of M2β-S1 per 30 plates of infected C2C12 cells. The M2β-S1 was 95% pure based on Coomassie-stained SDS-polyacrylamide gels (Fig. 1). We assumed the two low molecular weight bands, which co-purified with the M2β-S1 heavy chain (99,021 kDa), corresponded to the myosin light chains. The low molecular weight bands were excised from the gel and digested into peptides using trypsin, and their sequences were determined using LC-MS/MS. The upper band generated unique peptides from two essential light chain isoforms as follows: nine peptides corresponding to 65% of the myosin light chain 1/3 (Myl1) sequence, skeletal muscle isoform (UniProt P05977), and 13 peptides corresponding to 55% of the myosin light chain 4 (Myl4) sequence, atrial/fetal isoform (UniProt P09541). The lower band generated 12 highly abundant unique peptides corresponding to 70% of the sequence of myosin regulatory light chain 2 (Myl6), skeletal muscle isoform (UniProt P07457). The mouse essential light chains Myl1 and Myl4 were 75 and 79% identical and 83 and 91% similar, respectively, to the human cardiac muscle essential light chain (Myl3) (UniProt P08590). The mouse regulatory light chain contains 74% sequence identity and 87% sequence similarity with the human cardiac muscle regulatory light chain (Myl2) (UniProt P10916) (see supplementary Fig. S1 for alignments).

**Actin-activated ATPase of M2β-S1**—The maximum actin-activated ATPase rate and actin dependence of the ATPase rate of M2β-S1 were not significantly impacted by the presence of DMSO (0.1%) (Fig. 2A and Table 1). Biotinylation of the Avi tag of M2β-S1 also did not alter the ATPase activity. The presence of 10 \(\mu\)M OM reduced the maximal ATPase \(k_{cat}\) 4.5-fold and dramatically reduced the actin concentration at which ATPase is half-maximal \((K_{ATPase})\) 30-fold. The OM-induced inhibition of the actin-activated ATPase was evaluated in a concentration-dependent manner to determine the EC_{50} (0.52 ± 0.10 \(\mu\)M) (Fig. 2B).

**Actin Co-sedimentation Assays with M2β-S1**—We examined the steady-state actin affinity of M2β-S1 using actin co-sedimentation assays (Fig. 2C). The experiments were performed using conditions nearly identical to the ATPase assays with the exception that the co-sedimentation assay was performed over a longer time period (\(~10\) min). The actin-affinity of M2β-S1 in

and does not alter skeletal or smooth muscle myosin (6). Muscle fiber studies that have examined the impact of OM are somewhat contradictory. A study that utilized rat cardiac muscle found enhanced contractility (fractional shortening) without changes in the calcium transient (6). Other studies have revealed an increase in calcium sensitivity as measured by the force-\(p\)Ca relationship in mouse and rat myocardium, but a slowing of force development and relaxation was also noted (21, 22). Only one study has examined human myocardium and found a slowing of cross-bridge kinetics and an increase in sensitivity to calcium (23).

To investigate how OM impacts cardiac myosin motor performance, studies have examined purified cardiac myosin using steady-state and transient kinetic analysis as well as in vitro motility assays (6, 24). A study on porcine cardiac myosin determined that OM alters the hydrolysis equilibrium constant to favor products and enhances the rate of actin-activated phosphate release, but it does not change the ADP release step (24). These factors were predicted to enhance the number of myosin cross-bridges in the strongly bound state, which would explain the increased force in muscle fiber studies. However, OM was found to decrease the maximum actin-activated ATPase rate (\(~2\)-fold) (24), but the step(s) in the ATPase cycle slowed by OM were not determined. Interestingly, it was found that OM dramatically reduces (10–20-fold) the sliding velocity in the in vitro motility assay (24–27). These results were puzzling because the study by Liu et al. (24) found no change in the ADP release rate constant, and this step correlates with sliding velocity in a detachment limited model of actomyosin motility. Thus, it has been proposed that the increase in the number of actin-bound cross-bridges enhances the load experienced by the force generating cross-bridges and slows the ADP release rate constant and hence sliding velocity (24). The ADP release step has indeed been demonstrated to be load-sensitive and responsible for the load-dependent changes in the ATPase rate in contracting muscle (28, 29). Therefore, further investigation of specific step(s) in the ATPase cycle that are slowed by OM is required to define the complete kinetic mechanism. In addition, it is important to directly measure the duty ratio, the fraction of the ATPase cycle myosin is bound to actin, in the in vitro motility assay to evaluate hypotheses about how OM dramatically slows sliding velocity. Finally, it is important to examine the impact of OM on human cardiac myosin as well as human cardiac muscle to allow direct correlations to clinical studies.

In this study, we investigated the mechanism of action of OM on recombinant human \(\beta\)-cardiac myosin using steady-state and transient kinetic measurements as well as in vitro motility assays. We also studied the impact of the drug on the muscle mechanics of human myocardium. We find that OM dramatically alters the myosin ATPase kinetics, which creates enhanced drag forces that contribute to the slowing of sliding velocity. Based on measurements with permeabilized human cardiac muscle, we find that OM increases calcium sensitivity and slows force generation, but it does not change steady-state force at saturating calcium concentrations.
the presence of ATP was similar in the presence and absence of OM (\(K_{\text{act in}} = 145 \pm 18\) and \(155 \pm 13 \ \mu M\), respectively).

**Transient Kinetic Analysis of Actin-activated Product Release**—We examined the ADP release rate constant by mixing the actomyosin-mantADP complex (0.5 \(\mu M\) \(M2\beta-S1\), 0.6 \(\mu M\) actin, 10 \(\mu M\) mantADP) with saturating ATP (1 mM), and we found that there was no difference in the presence and absence of OM (\(k_{\text{obs}} = 313 \pm 6\) and \(306 \pm 5\) s\(^{-1}\), respectively) (data not shown). We also performed single turnover sequential mix experiments in the stopped flow to monitor the actin-activated product release steps of \(M2\beta-S1\) in the presence and absence of OM (Fig. 3). The experiment was performed by mixing \(M2\beta-S1\) with substoichiometric mantATP, aging the reaction for 10 s to allow ATP binding and hydrolysis, and then mixing with varying concentrations of actin (0.25 \(\mu M\) \(M2\beta-S1\), 0.23 \(\mu M\) mantATP, varying actin concentrations). We observed a two-exponential fluorescence transient at each actin concentration both in the presence and absence of OM (Fig. 3, \(A-D\)). The rate constant of the slow phase was independent of actin concentration and relatively similar in the presence and absence of OM (0.1–0.6 s\(^{-1}\)). The fast phase was hyperbolically dependent on actin concentration, and the maximum rate saturated at a much lower actin concentration in the presence of OM. In addition, the maximum rate constant of the fast phase was reduced in the presence of OM (4.3 \(\pm 0.5\) s\(^{-1}\)) compared with the absence (\(\approx 11.2 \pm 0.1\) s\(^{-1}\)). The relative amplitudes of the fast and slow phases were dependent on actin concentration with the fast phase more pronounced at a higher actin concentration (Fig. 3C). At high actin concentrations (40 \(\mu M\)), the relative amplitude of the slow phase was increased in the presence of OM (30%) compared with the absence (10%). Thus, the presence of OM slows actin-activated product release by reducing the rate constant of the fast pathway and increasing the flux through the slower product release pathway.

**In Vitro Motility of M2\beta-S1**—The sliding velocity produced by \(M2\beta-S1\) in the *in vitro* motility assay was evaluated in the presence of DMSO and 10 \(\mu M\) OM by examining three separate protein preparations at a loading concentration of 0.48 \(\mu M\) (Fig. 4). There were slight differences in the average sliding velocity (50 filaments) determined in each preparation for the DMSO (1646 \(\pm 30, 1837 \pm 36,\) and \(1469 \pm 22\) nm/s) and OM (7.87 \(\pm 0.14, 6.50 \pm 0.22,\) and \(6.23 \pm 0.19\) nm/s) conditions. Control experiments performed in the absence of ATP demonstrated no movement during a 10-min acquisition (data not shown), which confirmed the sensitivity of the velocity measurements. Therefore, the data from all three preparations was pooled together (150 filaments) to determine the average sliding velocity in the presence of DMSO (1651 \(\pm 24\) nm/s) or OM (6.87 \(\pm 0.12\) nm/s). The average velocity of \(M2\beta-S1\) was similar in the

---

**FIGURE 1.** *Purified recombinant human M2\beta-S1*. Representative SDS-polyacrylamide gel of purified recombinant \(M2\beta-S1\) compared with molecular weight standards as indicated. The myosin heavy chain (HC), regulatory (RLC), and essential light chains (ELC) are labeled.

**FIGURE 2.** *Steady-state ATPase activity and actin affinity of M2\beta-S1 in the presence and absence of OM.* A, ATPase activity of \(M2\beta-S1\) was examined as a function of F-actin concentration in MOPS20 buffer in the presence of 0.1% DMSO, 10 \(\mu M\) OM, or buffer only. The ATPase activity data were fit to a hyperbolic function to determine \(K_{\text{act in}}\) and \(k_{\text{ATPase}}\), ATPase activity in the presence of 40 \(\mu M\) actin was examined in the presence of varying concentrations of OM. The data were fit to Equation 1 to determine the EC_{50}. C, steady-state actin affinity was measured in the presence and absence of OM using the actin co-sedimentation assay. The fraction of \(M2\beta-S1\) bound to actin is plotted as a function of actin concentration and fit to a hyperbolic equation to determine \(K_{\text{act in}}\), Errors bars represent standard deviation of the mean from three protein preparations (some error bars are smaller than the symbol and therefore are not shown).


### TABLE 1

Summary of actin-activated ATPase results with M2β-S1

<table>
<thead>
<tr>
<th>Conditions</th>
<th>V₀</th>
<th>kₐt</th>
<th>K_{ATPase}</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM KCl</td>
<td>0.02 ± 0.02</td>
<td>7.89 ± 0.56</td>
<td>69.88 ± 7.11</td>
</tr>
<tr>
<td>20 mM KCl + Biotinylated M2 β</td>
<td>0.01 ± 0.01</td>
<td>7.36 ± 0.56</td>
<td>58.75 ± 5.59</td>
</tr>
<tr>
<td>20 mM KCl + DMSO</td>
<td>0.02 ± 0.01</td>
<td>7.48 ± 0.17</td>
<td>56.53 ± 13.92</td>
</tr>
<tr>
<td>150 mM KCl + DMSO</td>
<td>0.01 ± 0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20 mM KCl + OM</td>
<td>0.04 ± 0.01</td>
<td>1.65 ± 0.05</td>
<td>1.91 ± 0.44</td>
</tr>
<tr>
<td>150 mM KCl + OM</td>
<td>0.01 ± 0.01</td>
<td>1.46 ± 0.05</td>
<td>95.31 ± 63.0</td>
</tr>
</tbody>
</table>

† M2 β-S1 is in MOPS 20 Buffer.  ‡ Biotinylated M2-β-S1 is in MOPS 20 Buffer.  § M2 β-S1 in MOPS (150 mM KCl) Buffer is in the presence of 0.1% DMSO.  ¶ M2 β-S1 in MOPS 20 Buffer is in the presence of 10 µM OM.  ‖ M2 β-S1 in MOPS (150 mM KCl) Buffer is in the presence of 10 µM OM.

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**Impact of Omecamtiv Mecarbil on Cardiac Myosin**

this hypothesis by measuring the in vitro motility as a function of increasing ionic strength (20–150 mM KCl) in two protein preparations, because the weak binding states are known to be highly sensitive to ionic strength (Fig. 5 and Table 2 (30–32)). We found that in the absence of OM, the increasing ionic strength results in little change in the velocity up to 100 mM KCl and a small reduction in velocity at 150 mM KCl (Fig. 5A). In the presence of OM, ionic strength increased the sliding velocity as indicated by more than a 2-fold increase demonstrated when comparing 20 and 150 mM KCl (Fig. 5B). We also found that the ATPase activity in the presence of 40 mM actin was more sensitive to ionic strength in the absence of OM, but in the presence of OM the ATPase was much less sensitive to the increase in ionic strength (Fig. 5C). In the presence of OM, we were able to determine the maximum ATPase activity at high ionic strength (150 mM KCl), although this was not feasible in the absence of OM (Table 1). Because the maximum ATPase rate was similar at high and low ionic strength, the enhanced sliding velocity observed at higher ionic strength is at least partially due to the attenuation of drag forces from weakly bound myosin heads.

**Density Dependence of In Vitro Motility**—We examined the density dependence of the sliding velocity by performing the in vitro motility assay as a function of M2β-S1 concentration loaded into the flow cell. At low ionic strength (MOPS20 buffer) in the absence of OM, we observed little change in the density dependence, but in the presence of OM, the velocity increased 2.5-fold as the loading concentration was decreased from 2.0 to 0.24 µM (Fig. 6, A and B). At higher ionic strength (100 mM KCl) in the absence of OM, the velocities gradually decreased as density was decreased, whereas in the presence of OM, the velocity increase at lower densities was attenuated compared with low ionic strength conditions.

**Duty Ratio Measurements**—To evaluate the hypothesis that OM increases the duty ratio, we attempted to directly examine the duty ratio in the in vitro motility assay (100 mM KCl) by examining the velocity as a function of available myosin heads on the surface (Fig. 7, A and B). We determined the number of heads on the motility surface using NH₄-ATPase assays (38 heads and 117 heads per µm of actin filament at 0.24 and 0.35 µM loading concentrations, respectively). We found it difficult to obtain sliding velocities at low motor density (≤10 available heads), and thus fitting the data to established equations (33, 34) was not robust. We did observe a trend in the data that demonstrated a relative increase in duty ratio in the presence of OM compared with DMSO. We also evaluated the duty ratio by plotting the velocity as a function of actin filament length at 0.24 µM loading concentration, and a similar trend was observed (Fig. 7B).

**Muscle Mechanics of Human Myocardium**—To evaluate the effects of OM on the contractile properties of human myocardium, we performed experiments using chemically permeabilized multicellular preparations. Maximum isometric force was not significantly altered by OM (Fig. 8A). In contrast, 1 and 10 µM OM significantly increased pCa₉₀, indicating that less free Ca²⁺ was required to produce half-maximal Ca²⁺ activation (Fig. 8B). These concentrations of OM also slowed kᵣ, the rate of tension recovery following a rapid shortening/re-stretch perturbation (Fig. 8C). These data suggest that OM reduces the...
rate at which cross-bridges transition to force-dependent states in a dose-dependent manner.

**Discussion**

The discovery of drugs that directly interact with myosin to alter the force and velocity properties of cardiac muscle are promising, because they may be able to improve contractile performance without altering calcium homeostasis and myocardial oxygen demand. Our characterization of the motor properties of M2β-S1 suggests there are two kinetic pathways in the presence of OM. The drug can enhance the duty ratio of myosin heads that flux through the conserved ATPase pathway by increasing the period of time they are strongly bound to the actin filament during filament sliding. However, OM also traps some of the myosin heads in a weakly bound state with slow product release. We demonstrate that the main impact of OM

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**TABLE 2**

Summary of ionic strength dependence of *in vitro* motility assay results with M2β-S1

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Average sliding velocity (nm/s (±stderr))</th>
<th>No. of filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DMSO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM KCl</td>
<td>1783 ± 37</td>
<td>60</td>
</tr>
<tr>
<td>35 mM KCl</td>
<td>1839 ± 35</td>
<td>60</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>1739 ± 36</td>
<td>60</td>
</tr>
<tr>
<td>75 mM KCl</td>
<td>2074 ± 49</td>
<td>60</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>1817 ± 22</td>
<td>60</td>
</tr>
<tr>
<td>150 mM KCl</td>
<td>1284 ± 43</td>
<td>30</td>
</tr>
<tr>
<td><strong>OM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM KCl</td>
<td>7.33 ± 0.18</td>
<td>60</td>
</tr>
<tr>
<td>35 mM KCl</td>
<td>9.39 ± 0.35</td>
<td>60</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>9.76 ± 0.25</td>
<td>60</td>
</tr>
<tr>
<td>75 mM KCl</td>
<td>13.07 ± 0.53</td>
<td>60</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>14.43 ± 0.20</td>
<td>60</td>
</tr>
<tr>
<td>150 mM KCl</td>
<td>17.17 ± 0.18</td>
<td>30</td>
</tr>
</tbody>
</table>
on human cardiac muscle mechanics is a shift in calcium sensitivity that is likely caused by the enhanced duty ratio that increases cooperative activation of the thin filament. In addition, OM slows down the kinetics of force development without changing steady-state force at maximum calcium concentrations.

Impact of OM on the Myosin ATPase Cycle in Solution—Our results add to the previous transient kinetic analysis of porcine cardiac heavy meromyosin and bovine cardiac myosin S1 in the presence of OM (6, 24). Similar to the previous findings, we found that when ADP release is measured by mixing a complex of actomyosin-ADP with excess ATP, the determined rate constant is nearly identical in the presence and absence of OM. This method likely monitors the final step of ADP release from actomyosin ($K'_{5A}$). We also examined actin-activated product release with sequential mix experiments and provide evidence for two product release pathways. The actin dependence of the rate constants of the fast phase from the sequential mix experiments (Fig. 3A, 70% of the amplitude at saturating actin) is very similar to the steady-state ATPase results (Fig. 2A). Thus, the fast phase likely represents the traditional pathway (Scheme 1, blue arrows) in which the M-ADP-P complex binds to actin and releases phosphate ($K'_{4A}$) and then ADP ($K'_{5A}$ and $K'_{5B}$). We propose that the presence of OM slows the flux through the traditional pathway by slowing the isomerization between actomyosin-ADP states ($K'_{5A}$). The actin dependence of the ATPase assay ($K'_{ATPase}$) is thought to be mainly determined by the hydrolysis equilibrium constant as well as the rate constants for actin-activated phosphate release and ADP release (35). We find that the reduction in $K'_{ATPase}$ in the presence of OM is consistent with the previous transient kinetic studies (6, 24) and our current results that suggest $K'_{5A}$ is reduced in the presence of the drug.

The slower pathway observed in our sequential mix experiments may be associated with slow ATP hydrolysis ($K_s$) or phosphate release ($K_p$). The slower pathway could be associated with the formation of strong or weak actin binding states, but because the actin co-sedimentation assay demonstrates that the steady-state actin affinity is unchanged by OM, we favor the latter. A previous transient kinetic study proposed a branched pathway associated with formation of a post-hydrolysis dead end intermediate with slow phosphate release (24). In this model, ATP hydrolysis occurs before formation of the pre-power stroke state, which leads to formation of myosin with hydrolyzed ATP in its active site but still in a post-power stroke state. We propose that OM increases the time that myosin spends in this pathway and stabilizes the dead end intermediate that generates a pool of non-force generating heads. The proposed mechanism is consistent with the increased drag forces that are ionic strength-dependent in the motility assay. Our transient kinetic results are consistent with the 4–5-fold decrease in the maximum steady-state ATPase rate. Further transient kinetic analysis is necessary to directly examine the impact of OM on key steps in the ATPase pathway, including the affinity for actin in the weak binding states and the isomerization between actomyosin-ADP states ($K'_{5A}$).

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FIGURE 7. Duty ratio of M2β-S1 in the presence and absence of OM. A, we examined the duty ratio in the presence of DMSO and OM in high ionic strength buffer (100 mM KCl) by examining the sliding velocity as a function of available myosin heads (loading concentration was 0.24 or 0.35 μM). The number of available myosin heads was dependent on the density (determined with NH₄-ATPase assays) and the actin filament length. The trend in the data demonstrates a higher duty ratio in the presence of OM. B, velocity as a function of actin filament length (0.24 μM loading concentration) was also plotted, and the data are fit to a hyperbolic function to highlight the relative difference between the two conditions.

Indeed, a mouse model that induced overexpression of the atrial ELC isoform in the atrium resulted in increased shortening velocity (1.78-fold) in isolated muscle and sliding velocity in
the in vitro motility assays (1.17-fold) (42). Therefore, the differences in the velocities observed in this work compared with previous studies could be due to the methods of attaching the myosin to the surface, the length of the lever arm in the constructs examined, the light chain composition, and slight differences in the ionic strength and temperature utilized.

The large decrease in sliding velocity observed in the presence of OM in this study is more pronounced than the previous work with porcine and human cardiac myosin (15–20-fold) (24, 26, 27). One possible explanation for these differences is that at the higher velocities we observed in the absence of OM allowed for a larger OM-induced decrease to be observed as was found with α-cardiac myosin (26). However, the mechanism of how OM reduces the sliding velocity in the motility assay is still controversial. Our results suggest one major factor that could account for the large decrease in velocity in the presence of OM is the proposed reduction in the ADP isomerization step (K_5A), which would increase the time myosin is attached to actin (t_om).

If we assume a detachment limited model of sliding velocity (V = d_{uni}/t_om) (15) and no change in the unitary displacement (d_{uni}), then the velocity in the presence of OM is reduced proportionately to t_om. Therefore, it is important to examine d_{uni} and the rate-limiting step in the M2β-S1 ATPase cycle to further evaluate this argument. Another possibility that has been proposed (24–26) is that OM increases the t_om of force generating cross-bridges by a strain-dependent ADP release mechanism. In this mechanism there is an internal load placed on the actin filament because of myosin heads that have completed their power stroke but are still attached to actin. The attached time of the force generating cross-bridges increases because they have to work against the internal load and adapt by slowing their ADP release rate constant. However, there is currently no direct evidence for changes in strain-dependent ADP release in the presence of OM.

We demonstrate the density dependence of in vitro motility is very unusual in the presence of OM, especially at low ionic strength. The trend of the increase in velocity at lower densities suggests that with fewer heads available there is a reduction in the drag forces that slow the velocity. These drag forces could come from post-power stroke strongly bound heads or weakly bound non-force generating heads. The weakly bound states are strongly affected by increases in ionic strength, which we demonstrate attenuates the OM-induced inhibition of actin sliding. Thus, our results suggest that the inhibition by OM can be attenuated by reducing the number of heads available to interact with the actin filament, which can be altered by changing the ionic strength or the motor density. Our results are consistent with a population of myosin heads trapped in a weak actin binding state that contributes to the drag forces that slow actin filament sliding. However, the sliding velocities observed at the highest ionic strength measured did not return the velocity to the values in the absence of OM. Thus, we propose that both the increase in t_om of force generating heads, by strain-dependent and/or non-strain-dependent mechanisms and drag forces from weakly bound non-force generating heads contribute to the reduced in vitro motility sliding velocity in the presence of OM.

Impact of OM on Cardiac Muscle Mechanics—Studies of the impact of OM on cardiac muscle mechanics are conflicting, but have some common findings. The initial studies fit well into a hypothesis that OM increases the rate of transition into the strong binding states and explains the enhanced force observed in the corresponding muscle fiber studies (6). Further studies on rat myocardium demonstrated an increase in calcium sensitivity with little change in steady-state force development and slowed relaxation (22). Recent studies have examined the impact of OM on human myocardium and found that OM increases isometric force at sub-maximal calcium concentrations and slows cross-bridge detachment and recruitment (23). Our study adds to these studies by further examining the impact of OM on human myocardium in a dose-dependent manner. We find an increase in calcium sensitivity with little change in steady-state force at maximum calcium concentrations. In addition, we observe a decrease in the rate of force development. We propose that the rate of force development could be altered by the weakly bound non-force generating cross-bridges or slow detachment of cross-bridges due to a slower ADP isomerization. The ionic strength of the muscle fiber experiments was slightly higher (180 mM) than the highest ionic strength in motility measurements where we still observed a large inhibition of the sliding velocity. Overall the muscle mechanic studies demonstrate that OM may help enhance force at lower calcium concentrations, whereas OM also has the potential to slow force development and reduce shortening velocity.
The results from clinical trials demonstrate that OM can improve systolic ejection time and stroke volume (18). However, end diastolic volume was reduced, which is in agreement with an impact on slowed relaxation kinetics. In addition, some patients experienced ischemia at higher doses. A larger phase II clinical trial found OM was well tolerated and confirmed previous reports of increases in systolic ejection time, although there was no improvement in the primary end point of dyspnea (20). Another study suggests OM causes an increase in oxygen consumption, which could impact bioenergetics in long term treatment regimens (43). Overall, it appears that dosing could be critical to receive the maximum benefits of the OM-induced increase in force at submaximal calcium without impacting the rate of force development and shortening velocity.

Summary—We have performed a thorough analysis of human M2β-S1 in the presence and absence of OM using both in vitro motility and solution ATPase studies. We complement this work with an investigation of the impact of OM on human cardiac muscle mechanics. Our results support a model that suggests OM slows ATPase cycle kinetics by slowing the transition between actomyosin-ADP states which results in an increase in duty ratio. OM also results in a population of crossbridges trapped in a weak actin binding state with slow product release. The enhanced duty ratio increases the time period that myosin heads are attached to the thin filament in cardiac muscle which cooperatively activates the thin filament, enhances calcium sensitivity, and increases force at low calcium without changing steady-state force at maximum calcium. The negative impact of OM appears to be that it slows force development and could slow shortening velocity and may cause ischemia at high doses. Despite these potential drawbacks, an appropriate dose may provide a critical boost to contractile force in systolic heart failure patients. In addition, OM is an interesting proof of concept drug that may pave the way for future treatments that directly interact with contractile proteins to enhance the force and velocity properties of cardiac muscle.

**Experimental Procedures**

Reagents—ATP and ADP were prepared from powder (44). Omecamtiv mecarbil (CK-1827452) was purchased from Selleck Chemicals or AdooQ Bioscience. Omecamtiv mecarbil was dissolved in DMSO at a concentration of 10 mM. All solution experiments were performed in MOPS20 buffer (10 mM MOPS, pH 7.0, 20 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT) with the addition of appropriate KCl concentrations as indicated. 2'-Deoxy-ADP and 2'-deoxy-ATP labeled with N-methylanthraniloyl at the 3’-ribose position (mantADP and mantATP, respectively) were purchased from Jena Biosciences.

Construction of Expression Plasmids—The human cardiac myosin cDNA (AA5187.1) was purchased from Thermo Fisher Scientific. PCR amplification was used to subclone the M2β cDNA (AAA5187.1) was purchased from Thermo Fisher Scientific. The pAd.M2β-S1 was transformed into XL-10 Gold cells for amplification, and the pAd.M2β-S1 DNA was digested with Pac1 and transfected into Ad293 cells to allow for virus packaging and amplification. The Ad293 cells were grown in DMEM supplemented with 10% fetal bovine serum. The large scale virus preparation was performed by infecting 60 plates (145 mm diameter). The virus was harvested with freeze-thaw cycles followed by CsCl density sedimentation. The final virus titers were typically 10¹⁰ to 10¹¹ pfu/ml. C₂C₁₂ cells grown to 90% confluence in DMEM supplemented with 10% fetal bovine serum (typically 20–30 145-mm diameter plates) were differentiated by changing the media to DMEM supplemented with 10% horse serum and 1% fetal bovine serum. The C₂C₁₂ cells were infected with recombinant adenovirus (5 × 10⁸ pfu/ml) diluted into differentiation media. The media were changed after 2 days, and cells were harvested on day 7. The cells were lysed with a 50-mL Dounce in lysis buffer (50 mM Tris, pH 7.0, 200 mM KCl, 2 mM ATP, 1 mM ATP, 0.5% Tween 20, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 1 mM PMSF) and spun two times for 15 min at 25,000 rpm in a T50 rotor at 4 °C. The supernatant was added to a 1-mL anti-FLAG M2 resin column, washed with wash buffer (10 mM Tris, pH 7.5, 200 mM KCl, 1 mM EGTA, 1 mM EDTA, 2 mM MgCl₂, 2 mM ATP, 1 mM DTT, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 1 mM PMSF), and eluted with wash buffer containing FLAG peptide (0.167 mg/ml). The eluted M2β-S1 was subsequently precipitated with ammonium sulfate and dialyzed into MOPS20 buffer overnight at 4 °C. M2β-S1 was biotinylated for in vitro motility studies by incubating M2β-S1 with BirA (10 μg/ml) for 1 h at 25–30 °C, and subsequently precipitated with ammonium sulfate and dia lyzed into MOPS20 buffer overnight at 4 °C (46).

M2β-S1 purity was assessed by Coomassie-stained SDS-polyacrylamide gels, and protein concentration was determined by Bradford assay using BSA as a standard. Similar results were obtained by measuring the absorbance and using the predicted extinction coefficient (ε₂₉₀ = 1.38 × 10⁸ M⁻¹ cm⁻¹). Actin was purified from rabbit skeletal muscle using an acetone powder method (47). The actin concentration was determined by absorbance at 290 nm (ε₂₉₀ = 2.66 × 10⁴ M⁻¹ cm⁻¹). A molar equivalent of phalloidin was added to stabilize F-actin.

Mass Spectrometry—Expression of M2β-S1 light chain isoforms was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The myosin light chains associated with the M2β-S1 were separated on SDS-polyacrylamide gels and stained by Coomassie. The two resultant low molecular mass bands (~22 and 19 kDa) were excised from the gel, destained with 50% acetonitrile, and digested with 1 μg of trypsin (Promega) in 50 mM ammonium bicarbonate as described (48). The resultant peptides were reconstructed in 0.05% heptafluorobutyric acid and separated on an Acquity UPLC HSS T3 column (100 Å, 1.8 μm, 1 × 150 mm) (Waters) attached to a Dionex UltiMate 3000 HPLC (Dionex). The HPLC effluent was directly injected into a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer through an electrospray ionization source (Thermo Fisher Scientific). Data were collected in data-dependent MS/MS mode with the top five most abundant ions.
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being selected for fragmentation. Peptides were identified from the resultant MS/MS spectra by searching against a mouse database (downloaded from UniProt 2/15) using SEQUEST for running the Proteome Discoverer 2.1 software (Thermo Fisher Scientific). Peptide oxidation was accounted for by addition of 15.99 and 31.99 Da to each methionine residue. All MS/MS fragmentation spectra were manually confirmed. The area under each deisotoped LC peak was determined using Proteome Discoverer 2.1 (Thermo Fisher Scientific).

**Steady-state ATPase Activity**—Steady-state ATP hydrolysis by M2β-S1 (100 nM) in the absence and presence of actin (0–60 μM) was examined by using the NADH-linked assay (49–52) with a final MgATP concentration of 1 mM. The assay was performed in an Applied Photophysics stopped-flow apparatus (Surrey, UK) in which the NADH absorbance at 340 nm was monitored continuously for 200 s. The ATPase rate at each actin concentration was determined, and the Michaelis-Menten equation \( (V_{o} + (k_{\text{cat}} \cdot [\text{actin}])/(K_{\text{ATPase}} + [\text{actin}])) \) was used to determine \( k_{\text{cat}} \) and \( K_{\text{ATPase}} \), where \( V_{o} \) is the ATPase rate in the absence of actin; \( k_{\text{cat}} \) is the maximal ATPase rate, and \( K_{\text{ATPase}} \) is the actin concentration at which the ATPase activity is half-maximal. The data at each actin concentration represents an average of 2–3 protein preparations.

**Actin Co-sedimentation Assay**—The steady-state actin affinity of M2β-S1 in the presence of ATP was measured using an actin co-sedimentation assay. M2β-S1 (0.2 μM) was equilibrated with various actin concentrations (0–60 μM) in MOPS20 buffer containing the ATP regeneration system (20 units ml/1 pyruvate kinase and 2.5 mM phosphoenolpyruvate). We added 1 mM ATP and immediately centrifuged the samples for 8 min at 25 °C (TLA.120.1 rotor at 120,000 rpm). The supernatant and pellet were examined by SDS-PAGE followed by Western blotting, and the biotinylated M2β-S1 was detected using a streptavidin-alkaline phosphatase conjugate. The intensity of the M2β-S1 bands was examined using ImageJ, and the fraction bound was determined using the equation pellet/(supernatant + pellet). The plot of fraction bound as a function of actin concentration was fit to a hyperbolic function to determine the steady-state actin affinity (\( K_{\text{actin}} \)).

**Transient Kinetic Studies**—The stopped-flow apparatus equipped with an excitation monochrometer (2-nm band pass filter) and appropriate emission filters was used to examine key steps in the actomyosin ATPase cycle of M2β-S1. The mant-labeled nucleotides were excited at 290 nm, and the emission was measured with a 395-nm long pass filter. The fluorescence transients were fitted using custom software provided with the instrument or with GraphPad Prism, and errors are reported as standard errors of the fit. All concentrations listed are final unless otherwise noted.

**In Vitro Motility Assay**—The actin filament sliding assay was performed as described previously (53) except for the method of adhering the myosin to the surface. Microscope coverslips were coated with 1% nitrocellulose in amyl acetate (Ladd Research). The surface was coated with streptavidin (0.1 mg/ml) and blocked with BSA (1 mg/ml) before the addition of biotinylated M2β-S1 (loading concentration, amount added to the flow cell, was varied between 0.24 and 2.0 μM). Unlabeled actin (2 μM) followed by an ATP (2 mM) wash was used to prevent interactions with dead heads. Actin labeled with either rhodamine phalloidin (DS Red filter; excitation/emission: 545/620 nm) or Alexa (GFP filter; excitation/emission, 500/535 nm) was visualized by fluorescence microscopy. An activation buffer with an appropriate concentration of DMSO or OM was added to the flow cell to initiate motility. Activation buffer contained the following: MOPS20 buffer, 0.35% methylcellulose, 2.5 mM phosphoenolpyruvate, 20 units ml/1 pyruvate kinase, 0.1 mg ml/1 glucose oxidase, 5 mg ml/1 glucose, 0.018 mg ml/1 catalase, and 2 mM ATP. The slide was promptly viewed using a NIKON TE2000 microscope equipped with a ×60/1.4NA phase objective and a Perfect Focus System. Images were acquired every second for 3 min or every 15 s for 10 min in the absence and presence of OM, respectively, using a shutter-controlled Coolsnap HQ2-cooled CCD digital camera (Photometrics) binned 2 × 2. Temperature was maintained at 26 ± 1 °C and monitored using a thermocouple meter (Stable Systems International). Image stacks were transferred to ImageJ for analysis via MTrackJ (54) and corrected for drift using StackReg. The average velocity was determined by tracking actin filaments manually for each condition (e.g. the path of each filament was determined by tracking the filament on a frame-by-frame basis, which allowed determination of average velocity). The actin filament lengths were measured using the length tool in ImageJ.

The number of myosin heads on the motility surface was measured using an NH₄³⁻-ATPase assay (34, 55). We evaluated the number of moles of phosphate produced in a motility flow cell chamber and compared that with a standard curve of moles of phosphate produced per concentration of M2β-S1. To determine the number of heads available per μM of actin filaments, we assumed that M2β-S1 could interact with a 20-nm band of the actin filament (10 nm of either side of the filament). The plot of the relative velocity as a function of myosin heads available (33, 34) was utilized to demonstrate the relative difference in duty ratio in the presence of OM.

The plots of the relative ATPase and motility as a function of OM concentration were fit to Equation 1,

\[
y = \text{Bottom} + \frac{\text{Top-Bottom}}{1 + 10^{x - \log IC_{50}}}
\]

(Eq. 1)

where \( x \) = OM concentration, and \( y \) = relative ATPase activity or sliding velocity, and Bottom and Top are the associated plateaus.

**Human Tissue**—The cardiac samples used in this study were obtained from patients at the University of Kentucky who had end-stage heart failure using the procurement system described by Blair et al. (56). Briefly, through-wall sections of the distal anterior region of the left ventricle were obtained from hearts that had been explanted during a cardiac transplant. Samples weighing ~500 mg were dissected from these sections, placed in 2-ml cryogenic vials, and snap-frozen in liquid nitrogen. The vials were subsequently stored in the vapor phase of liquid nitrogen at −150 °C until use. The experiments described in this study used specimens isolated from the sub-endocardial region of eight patients. All procedures were approved by the
University of Kentucky Institutional Review Board, and the subjects gave informed consent.

Preparations and Experimental Setup—Multicellular preparations were obtained by mechanical disruption followed by chemical permeabilization using 1% v/v Triton X-100 detergent (57). Suitably sized preparations were attached between a force transducer (resonant frequency, 600 Hz; model 403, Aurora Scientific, Aurora, Ontario, Canada) and a motor (step time 0.6 ms; model 312B, Aurora Scientific) and stretched to a sarcomere length of 2.24 μm in a solution with a pCa (= −log10[Ca2+]) of 9.0. The mean length of the preparations was 1055 ± 229 μm. The cross-sectional area was 5.87 ± 2.42 × 10−8 m2 (estimated assuming a circular profile). Experiments were conducted at 15 ºC using SLControl software (58).

OM Preparation and Incubation of Samples—Solutions with pCa values ranging from 9.0 to 4.5 and OM concentrations of 0.1, 1.0, or 10 μM were made by adding suitable amounts of OM dissolved in DMSO. The final percentage of DMSO in every experimental solution was 0.67%. Preparations were immersed in a pCa 9.0 solution containing 0 (control), 0.1, 1.0, or 10 μM for at least 3 min between trials. The ionic strength was held constant at 180 mM in all solutions.

Mechanical Measurements—Pilot tests showed that OM was difficult to wash out of the multicellular preparations. Each preparation was therefore exposed to only one concentration of OM (0, 0.1, 1.0, or 10 μM). Specimens were initially activated in a solution with a pCa 9.0 of 9.0. Force was recorded until the rate of tension recovery, ktr, was measured by rapidly shortening the preparation by 20%, holding it at the short length for 20 ms, and then re-stretching the preparation to its original length. The ktr was subsequently calculated by fitting the portion of the force record immediately after the re-stretch with a single exponential function of the form F(t) = A + B(1 − exp(−ktr t)). In this equation, F(t) is the force at time t, and A and B are constants. Calcium sensitivity (pCa50) was determined by repeating the force measurements with different solutions that had pCa values in the range 9.0 to 5.0. pCa50 values were calculated by fitting the resulting data to a modified Hill equation of the form F = Fpas + FCa ([Ca2+]n/[Ca2+]n + [Ca2+so]) where Fpas is the force measured in pCa 9.0 solution; FCa is Ca2+ -activated force; n is the Hill coefficient, and [Ca2+so]n is the free Ca2+ concentration required to develop half the maximum Ca2+ -dependent force.

Statistics for Muscle Mechanics on Human Samples—Experiments were performed using a total of 46 multicellular preparations from eight hearts. Data were analyzed using linear mixed models that allowed for the fact that multiple samples were analyzed from each heart. Compound symmetry was assumed for the covariance structure, and post-hoc analyses were performed using Tukey-Kramer corrections. p values less than 0.05 were considered significant. Data are reported as mean ± S.E.

Author Contributions—C. M. Y. designed the research; A. M. S., W. T., C. A. B., C. M. F., W. C. U., M. J. P., and C. M. Y. performed the research; A. M. S., W. T., C. A. B., C. M. F., W. C. U., M. J. P., K. S. C., and C. M. Y. analyzed the data; A. M. S., C. A. B., W. T., M. J. P., K. S. C., and C. M. Y. wrote paper. All authors approved the final version of the manuscript.

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Omecamtiv Mecarbil Enhances the Duty Ratio of Human β-Cardiac Myosin Resulting in Increased Calcium Sensitivity and Slowed Force Development in Cardiac Muscle

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Figure S1: Sequence alignments of murine essential and regulatory light chains with the ventricular human cardiac isoforms. A) The two murine essential light chains present in our M2β-S1 construct, myosin light chain 1/3 (UniProt - P05977) and myosin light chain 4 (UniProt - P09541), were 75% and 79% identical and 83% and 91% similar, respectively, to the human myosin light chain 3 (P08590). B) The murine regulatory light chain present in our M2-βS1 construct, myosin regulatory light chain 2 (UniProt - P10916), is 74% identical and 87% similar to the human cardiac muscle regulatory light chain 2 (P10916). The sequences were aligned using Jalview with the similarity of each residue colored based on BLOSUM62 score.