Orthophosphate increases the efficiency of slow muscle-myosin isoform in the presence of omecamtiv mecarbil

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Omecamtiv mecarbil (OM) is a putative positive inotropic tool for treatment of systolic heart dysfunction, based on the finding that in vivo it increases the ejection fraction and in vitro it prolongs the actin-bond life time of the cardiac and slow-skeletal muscle isoforms of myosin. OM action in situ, however, is still poorly understood as the enhanced Ca2+-sensitivity of the myofilaments is at odds with the reduction of force and rate of force development observed at saturating Ca2+. Here we show, by combining fast sarcomere-level mechanics and ATPase measurements in single slow demembranated fibres from rabbit soleus, that the depressant effect of OM on the force per attached motor is reversed, without effect on the ATPase rate, by physiological concentrations of inorganic phosphate (Pi) (1-10 mM). This mechanism could underpin an energetically efficient reduction of systolic tension cost in OM-treated patients, whenever [Pi] increases with heart-beat frequency.
In the heart, mutations in the myosin motor are responsible for cardiomyopathies, which consist either in an increased contractility with reduced ventricular filling or diastolic dysfunction (hypertrophic cardiomyopathy, HCM) or in a decreased contractility with reduced ejection fraction or systolic dysfunction (dilated cardiomyopathy)\(^1\). A promising approach for systolic dysfunction treatment is the development of small molecules that, acting as cardiac myosin activators, are able to increase the contractility during the systole. Among them, omecamtiv mecarbil (OM) has been found to increase the systolic ejection fraction\(^2\)–\(^6\) and is currently in phase-three clinical trial\(^7\). OM acts on both a and β cardiac myosin heavy chain (MHC) isoforms and on the slow-skeletal MHC isoform\(^8\). Noteworthy, the β cardiac MHC isoform, which accounts for >95% of the myosin in the human ventricle, is encoded by the same gene (MYH7) as the slow-skeletal MHC isoform\(^8\)–\(^12\) and the two isoforms will be named β/slow MHC hereafter. OM binds to the catalytic domain of the myosin molecule between the nucleotide binding pocket and the converter domain, a communicating zone between the actin site and the lever arm responsible for the working stroke. As a consequence, the release of inorganic phosphate ([Pi]) is promoted and the lever arm of the molecular motor is stabilized in a pre-working stroke position.\(^2\)–\(^13\),\(^14\) Accordingly, the velocity of actin sliding in the in vitro motility assay is reduced by OM\(^15\)–\(^17\). A recent in vitro mechanical study\(^9\) has significantly advanced the understanding of OM mechanism, showing that OM binding to β-cardiac myosin inhibits the working stroke and prolongs the actin-attachment life time of the motor. In this way, in situ, OM may increase thin filament activation at low Ca\(^{2+}\) allowing recruitment of OM-free motors, able to generate force. However, a detailed in situ analysis of the effects of OM on the mechano-kinetics and energetic of myosin motors has still to be done.

Here we determine, in Ca\(^{2+}\)-activated demembranated fibres from rabbit soleus, the effects of OM on either the number and force of the attached myosin motors or their ATPase activity and how physiological concentrations of inorganic phosphate ([Pi]) influence these parameters. The slow-skeletal muscle of the rabbit has been chosen instead of the heart because their myosin isoforms exhibit similar affinity for OM\(^8\), while the required nanometre-microsecond resolution of sarcomere-level mechanics can only be achieved in demembranated myocytes from skeletal muscle\(^18\),\(^19\). Intact trabeculae dissected from the ventricle of the rat heart, on which sarcomere-level mechanics has been recently successfully exploited\(^20\),\(^21\), do not suit the present investigation because (i) only skinned myocytes allow the required manipulation of [Ca\(^{2+}\)] and [Pi] and (ii) only ~20% of the myosin is β/slow MHC isoform and the remaining ~80% is a MHC isoform\(^21\). Determining the fraction of motors recruited for actin interaction during Ca\(^{2+}\)-activated isometric contraction of demembranated soleus fibres allowed the demonstration that OM reduces the average force per attached motor but at low Ca\(^{2+}\) it increases the number of attached motors. The inhibitory effect of OM on force generation is reversed by increasing [Pi] (range 1–10 mM), which acts as an allosteric competitor that allows the OM-bound no-force-generating motors to release OM and re-enter the force-generating cycle without any change in the rate of ATP hydrolysis. We conclude that in the heart systole, in which the internal [Ca\(^{2+}\)] remains below the saturating level, the inotropic action of OM occurs via two mechanisms: the first is the increase in the number of attached motors, the second, emerging at physiological levels of intracellular Pi (1–10 mM\(^22\)–\(^28\)), is a Pi-dependent recovery of the force per motor. This effect of Pi on the OM-bound motors represents an energetically efficient inotropic response in patients under OM treatment, whenever the increase of heart-beat frequency increases [Pi]\(^27\),\(^28\).

**Results**

**Titration of OM effect on the isometric force development.** Following a temperature jump from 1 to 12 °C in the activating solution at saturating [Ca\(^{2+}\)] (pCa 4.5), the force rises from near zero to a steady value (T\(_0\)) with an almost exponential time course (Fig. 1a). Addition of 1 μM OM halves T\(_0\) and slows the force development that shows a sigmoïdal shape (Fig. 1b). In eight fibres T\(_0\) decreases from 146 ± 5 kPa in the control to 64 ± 3 kPa in the presence of 1 μM OM. The reciprocal of the half-time for force development, r\(_{TD}\), taken as an estimate of the rate of force development, is reduced by 1 μM OM from 3.7 ± 0.2 s\(^{-1}\) to 1.9 ± 0.1 s\(^{-1}\). The reduction of both T\(_0\) and r\(_{TD}\) by OM is dose-dependent as shown by the relations of T\(_0\) (Fig. 1c) and r\(_{TD}\) (d) versus [OM]. The effect nearly saturates at 10 μM OM when T\(_0\) is ~35 kPa and r\(_{TD}\) is ~1.4 s\(^{-1}\). The concentration of OM for half-maximal effect is ~0.5 μM for both T\(_0\) and r\(_{TD}\).

**OM does not alter the fraction of motors at full activation.** The half-sarcomere stiffness at T\(_0\) (k\(_0\)) during maximal Ca\(^{2+}\) activation is estimated from the slope of the T\(_i\) relation determined, both in the control and in the presence of OM (range 0.1–10 μM), by plotting the force attained in response to stepwise changes in fibre length against the corresponding changes in the half-sarcomere length measured with the striation follower (see "Methods") (Fig. 2a, b). The T\(_i\) relations are shown in Fig. 2c in both control (filled circles) and 1 μM OM (open circles). Both the ordinate intercept (T\(_0\)) and the abcissa intercept (Y\(_0\), an estimate of the half-sarcomere strain at T\(_0\)) reduce in OM (open circles) by a similar amount (~60%) relative to the control (filled circles). Thus, the slope of the T\(_i\) relation, k\(_0\), is the same in the presence of OM as in control. In the eight fibres used for these experiments, k\(_0\) is 28.1 ± 0.7 kPa nm\(^{-1}\) in control and remains almost constant independent of [OM] in the whole range of concentrations used (0.1–10 μM, Fig. 2d, triangles), with a mean value of 29.8 ± 0.6 kPa nm\(^{-1}\) (dashed line). In the same range of [OM], Y\(_0\) linearly depends on the isometric force and the extrapolation of the relation to zero force crosses the origin (Fig. 2e). This result indicates that the reduction of isometric force by OM at saturating [Ca\(^{2+}\)] is due to a proportional reduction in the strain of all the elastic elements in the half-sarcomere (myofilaments and attached myosin motors) and thus OM reduces the half-sarcomere force by reducing the average force per attached myosin motor without changing the number of attached motors.

This conclusion is supported quantitatively by extracting the stiffness of the array of attached motors, e\(_0\) (Fig. 2f), from k\(_0\), e\(_0\) is determined by subtracting the contribution of the filament compliance (C\(_f\)) from the half-sarcomere compliance (see "Methods"). The procedure is based on the definition of the half-sarcomere in terms of a simple mechanical model in which the attached motors, represented by an array of in parallel springs with average spring s\(_p\)\(^18\), are in series with the actin and myosin filament elasticity represented by a spring with an equivalent filament compliance C\(_f\). The values of C\(_f\) and s\(_p\) are reported in Table 1. Knowing C\(_f\) (15.2 nm MPa\(^{-1}\), average from Table 1), the dependence of e\(_0\) on [OM] can be calculated from the relation between k\(_0\) and [OM] (Eq. (1)). As shown in Fig. 2f, e\(_0\) remains almost constant (~55 kPa nm\(^{-1}\)) independent of [OM]. Under the sensible assumption that OM does not affect the stiffness of the motor, e\(_0\) is an estimate of the fraction of attached motors, β, which results constant independent of [OM]. On the other hand, in the presence of 1 μM OM s\(_p\) decreases from 3.20 to 1.57 nm (Table 1), indicating that the reduction of T\(_0\) to ~ 1/2 by 1 μM OM (Fig. 1c) is fully accounted for by a corresponding decrease in s\(_p\). T\(_0\) and the strain of all the elastic elements decrease in proportion in the whole range of [OM] (Fig. 2e), indicating that
Fig. 1 Effect of OM on isometric force development. Force development following T-jump (from 1 to 12 °C) in control (a) and in the presence of 1 μM OM (b) on an activated fibre (pCa 4.5). Traces show the length change per half-sarcomere (upper trace) and the force (lower trace). The artefact in the traces marks the transition to the test temperature drop. The horizontal line in the lower panels indicates zero force. Fibre length, 4.5 mm; length of the segment under the striation follower, 0.8 mm; average sarcomere length within the segment, 2.3 μm; CSA, 3000 μm²; pCa, 4.5. Dependence of the isometric force $T_0$ (c) and of the rate of force development $r_{TD}$ (d) on the concentration of OM. Lines, simulation with model of Fig. 6. Data are mean values ± SEM from eight fibres. Source data are provided as a Source Data file.

the reduction of $s_0$ accounts for that in $T_0$. The average force per attached motor ($F_0$) can be calculated from $s_0$ if the stiffness of the myosin motor ($\varepsilon$) is known: $F_0 = s_0 \cdot \varepsilon$. Taking $\varepsilon$ for the slow myosin isoform = 0.56 ± 0.04 pN nm⁻¹, $F_0$ is 1.8 ± 0.2 pN in control and 0.9 ± 0.1 pN in 1 μM OM (Fig. 3c).

OM increases myofilament Ca-sensitivity for motor attachment. As $[Ca^{2+}]$ decreases, the depressant effect of OM on $T_0$ also decreases. As shown in Fig. 3a, at pCa between 4.5 and 6.5, $T_0$ is lower in OM (open circles) than in control (filled circles), while at pCa > 7 $T_0$ is higher in OM. Fitting the data with the Hill equation shows that pCa50 (the pCa at which force attains half-maximum) increases from 6.57 in the control (continuous line) to 7.02 in the presence of OM (dashed line) (Table 2), indicating a corresponding increase in Ca²⁺-sensitivity of the force. At the same time the slope of the relation, estimated by the parameter $n$ of the Hill equation, which is an indicator of the degree of cooperativity in myofilament activation, decreases from 2.01 in the control to 0.84 in the presence of OM.

The stiffness of the array of attached motors $e_0$ (an estimate of $\beta$) and the average motor strain $s_0$ at each pCa can be calculated both in control and in 1 μM OM using Eqs. (1) and (2) as detailed in “Methods”. As shown in Fig. 3b, while at saturating $[Ca^{2+}]$ $e_0$ is the same with and without OM, at pCa larger than ~6.5 $e_0$ is higher in OM (open circles) than in control (filled circles). Instead the strain per motor $s_0$ and thus the force per motor $F_0$ are halved by the addition of 1 μM OM at saturating Ca²⁺+ (pCa 4.5) (Fig. 3c), but are not affected by the reduction of $[Ca^{2+}]$ in either the absence (filled circles) or the presence of OM (open circles).

The larger value of $\beta$ in OM at sub-saturating $[Ca^{2+}]$ but not at saturating $[Ca^{2+}]$ is the sign of a higher Ca²⁺+ sensitivity, which underpins OM as a potentiator of contractility. A more precise definition of the Ca²⁺+-dependence of this effect is given in Fig. 3d, where the difference ($\Delta T_0$) between the fitted $T_0$-pCa relations in 1 μM OM (dashed line in Fig. 3a) and in control (continuous line) is plotted versus pCa. At pCa 6.8, the reduced average force per motor (~½) in 1 μM OM is compensated by the increased fraction of attached motors (~twice); at pCa >6.8, 1 μM OM acts as a potentiator increasing the sarcomere force, because, while the force per motor is halved by 1 μM OM, the increased Ca-sensitivity gives a $\beta$ larger than twice the control.

Effect of Pi on isometric contraction with and without OM. The allosteric competition between binding of OM and the presence of Pi in the catalytic site of the motor has been studied by determining how, at pCa 4.5, the increase of [Pi] in 30 mM (where 1 mM is the [Pi] assumed for the ordinate intercept in Fig. 1d) and increases with the increase in [Pi] with a slope that progressively decreases. A similar Pi dependence of $r_{TD}$ is observed in the relation determined in 1 μM OM (Fig. 4b, open circles), beyond the depressant effect reported in Fig. 1d.
In the presence of 1 μM OM, the lack of the inhibitory effect of the increase in Pi on T₀ for [Pi] < 10 mM (Fig. 4a, open circles), while β decreases in the same way as in control (−38% at 10 mM Pi, Fig. 4c), results in the increase of the average motor strain s₀ from 1.41 ± 0.15 nm to 2.06 ± 0.06 nm (+46%, left ordinate in Fig. 4d, open circles). Above 10 mM Pi, Pi induces, also in the presence of 1 μM OM, comparable reduction of both T₀ and e₀ (open circles in Fig. 4a and c), so that s₀ remains roughly constant (open ordinate in Fig. 4d). F₀ increases from 0.79 ± 0.10 to 1.16 ± 0.09 pN with the increase in [Pi] in the range 1–10 mM (right ordinate in Fig. 4d, open circles) and then, for [Pi] >10 mM, remains roughly constant at ~1.1 pN, a value that anyway is still lower than in the absence of OM (~1.7 pN).

**OM and the rate of ATP hydrolysis in isometric contraction.** Faced with the contradictory or not exhaustive results on the OM effects on the ATPase activity15,29,35, the effect of the increase in [Pi] (range 1–20 mM) on the ATP hydrolysis rate (k_cat) in isometric contraction has been determined in the absence and in the presence of OM (Fig. 5a, b). In these experiments, in agreement with the mechanical experiments, in solution with no added Pi T₀ is reduced to ~50% the control value by the addition of 1 μM OM (circles in Fig. 5b). k_cat, instead, does not change significantly (triangles), being 0.055 ± 0.007 mM s⁻¹ in the absence of OM and 0.055 ± 0.005 mM s⁻¹ and 0.052 ± 0.006 mM s⁻¹ in 1 μM and 2 μM OM, respectively.

**Effect of Pi on ATP hydrolysis rate with and without OM.** The increase of [Pi] in the absence of OM reduces by the same amount T₀ (Fig. 5c, filled circles, data similar to those in Fig. 4a) and k_cat (Fig. 5d, filled circles). This finding is in agreement with previous work36. In the presence of 1 μM OM, the increase in [Pi] has an inhibitory effect on T₀ that emerges only at [Pi] above 10 mM (Fig. 5c, open circles, data similar to those in Fig. 4a, open circles). Instead, k_cat is reduced by the increase in [Pi] (Fig. 5d, open circles) in the same way as in the absence of OM (filled circles).

The tension cost of the isometric contraction (Eᵢ) can be calculated as the ratio of k_cat over T₀ (Fig. 5e). Without added Pi, Eᵢ in the presence of 1 μM OM is almost twice the control (points at 1 mM Pi in Fig. 5e, filled circle control, open circle 1 μM OM), as a consequence of the depressant effect of OM on the force per

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**Table 1 Estimates of the mechanical parameters of the half-sarcomere.**

|                  | Control            | 1 μM OM            |
|------------------|--------------------|--------------------|
| T₀ (kPa)         | 156 ± 7            | 69 ± 5             |
| Cₛ (nm MPa⁻¹)    | 15.2 ± 1.3         | 15.3 ± 2.0         |
| s₀ (nm)          | 3.20 ± 0.15        | 1.57 ± 0.10        |

Cₛ and s₀ are determined according to the procedure detailed in “Methods”, in control and in the presence of OM. The isometric force at saturating Ca²⁺ is reported in the first row. T₀, Cₛ, and s₀ slope and ordinate intercept from data in Fig. 7c. Source data are provided as a Source Data file.

**Table 2 Parameters of Hill’s equation fit to force-pCa relation in control and in the presence of OM.**

|                  | Control            | 1 μM OM            |
|------------------|--------------------|--------------------|
| n                | 2.01 ± 0.14        | 0.84 ± 0.15        |
| pCa⁰           | 6.57 ± 0.01        | 7.02 ± 0.07        |

n estimates the slope of the relation, pCa⁰, the pCa at which force attains half-maximum) estimates the Ca²⁺ sensitivity. Data are mean ± SEM from four fibres. Data from Fig. 3a.
Fig. 3 Effect of OM on myofilament Ca\(^{2+}\) sensitivity for motor attachment. a \(T_0\)-pCa relation in control (filled circles) and in the presence of 1 \(\mu\)M OM (open circles). Continuous (control) and dashed (1 \(\mu\)M OM) lines are calculated by fitting Hill equation \(T_0 = T_{0,45} \times (1 + 10^{(5\text{pCa} - \text{pCa}_{50})})/2\) to data. The best fit parameters are listed in Table 2. b Dependence of the stiffness of the motor array \(\varepsilon_0\) (left ordinate) and of the fraction of attached motors \(\beta\) (right ordinate) on \([\text{Ca}^{2+}]\). In control at saturating \([\text{Ca}^{2+}]\) is assumed 0.47. Continuous (control) and dashed (1 \(\mu\)M OM) lines are calculated by fitting the Hill’s sigmoidal equation to data. c Dependence of the strain per motor \(\varepsilon_0,\) (left ordinate) and of the force per motor \(F_0,\) (right ordinate) on \([\text{Ca}^{2+}]\). d Dependence of the difference \(\Delta T_0\) between the fitted \(T_0\)-pCa relation in 1 \(\mu\)M OM (dashed line in a) and in control (continuous line in a) on \([\text{Ca}^{2+}]\); dashed line indicates zero difference. In all panels, \([\text{Ca}^{2+}]\) is expressed in pCa units. Data are mean values ± SEM from four fibres. Source data are provided as a Source Data file.

Fig. 4 Effect of Pi on the isometric mechanical parameters with and without OM. Filled circles and continuous lines, control; open circles and dashed lines, in the presence of 1 \(\mu\)M OM. a Pi dependence of the isometric force \(T_0,\) expressed in units relative to the value without added Pi \((T_{0,0}).\) b Rate of force development \(r_{T0} \), c Stiffness of the motor array \(\varepsilon_0\) relative to the value without added Pi \((\varepsilon_{0,0}).\) d Motor strain \(s_0\) (left ordinate) and motor force \(F_0\) (right ordinate). In each panel: symbols, experimental data; lines, model simulation. Data are mean values ± SEM from three fibres. Source data are provided as a Source Data file.

Model simulation. A kinetic model able to explain the effects of OM and Pi on the in situ mechanics and energetics of the \(\beta/\)slow MHC isoform of myosin has been developed. The model is based on that originally proposed for fast skeletal muscle fibres from rabbit psoas, which has been modified to adapt the rate constants of the forward and backward transitions and the corresponding equilibrium constants \((k_{1,0}, k_{0,1}\) and \(K_{M}\) respectively) to fit the bulk of kinetic data from in vitro and in situ studies of \(\beta/\)slow MHC isoform in control condition (without added Pi and OM) (see Table 3 for the detailed justification of the assumptions made for each kinetic step and the dedicated section in Methods for the criteria of selection of the parameters for the model fitting). In particular, relevant kinetic constraints in this study are as follows: (i) \(r_{T0} \sim 4\) s\(^{-1}\) (Fig. 1d, ten times slower than that of fast muscle), (ii) \(\beta\) at \(T_0 = 0.47\) (40% larger than that of fast muscle) and (iii) \(k_{\text{cat}} = 0.055\) \(\text{mM}\) s\(^{-1}\) (ten times slower than that of fast muscle). The original model (green-highlighted part of the scheme in Fig. 6), in agreement with experimental evidence, assumes the formation of strongly bound, force-generating motors also before Pi release, so that rise in Pi should exert a minor inhibitory effect on \(T_0\) and \(\beta\) than on \(k_{\text{cat}}\). In contrast to this view, in fast skeletal muscle fibres Pi reduces \(T_0\) and \(\beta\) more than \(k_{\text{cat}}\), suggesting that the conventional chemomechanical cycle has to be integrated with a branch that allows motor detachment at an early stage of the motor \(F_0\) without effect on \(k_{\text{cat}}\). In the absence of OM \(E_T\) remains the same independent of [Pi] (filled circles), while in 1 \(\mu\)M OM (open circles) \(E_T\) decreases with the increase of [Pi] approaching the value in the absence of OM.
Fig. 5 Effect of OM on the rate of ATP hydrolysis in isometric contraction and its modulation by Pi. a Sample records from an activated fibre (pCa 4.5) of the time course of force and light absorbance (in absorbance units, a.u.) in the absence (left) and in the presence (right) of 1 μM OM. The dotted line on the absorbance trace is the 1st order regression equation fitted to the trace after force has attained a steady value. The slope of the regression is used as a measure of the ATP hydrolysis rate (k_{cat}). b Dependence on OM of T_0 (circles and dashed line) and k_{cat} (triangles and continuous line). Values are relative to the isometric values in the absence of OM (98 ± 17 kPa and 0.055 ± 0.007 mM s^{-1}, respectively). c Dependence on Pi of the isometric force (T_0) relative to the value without added Pi (T_{0,i}) in the absence (filled circles and continuous line) and in the presence (open circles and dashed line) of 1 μM OM. d Dependence on Pi of k_{cat} relative to the value without added Pi (k_{cat,1}). Symbols and lines as in (c). e Tension cost of the isometric contraction (E_T). Symbols and lines as in (c). E_T is normalized by the value in the absence of OM and no added Pi. b–e symbols, experimental data; lines, model simulation. Data are mean values ± SEM from 12 fibres in (b) and from four fibres in (c–e). Source data are provided as a Source Data file.

ATPase cycle (step 6), with Pi still bound to the catalytic site. The finding in this work that β and k_{cat} are reduced by the same extent by the rise in Pi (filled circles in Figs. 4c and 5d) confirms the requirements for the unconventional early detachment with the hydrolysis products still bound to the catalytic site.

To explain the effects of OM on the mechano-kinetics of the β/ slow isoform of the myosin motor in situ a branched pathway (pink-highlighted part in the scheme of Fig. 6) is required. In this way the scheme accounts for both the features present in the literature (points i, ii below) and those reported in this work (points iii–v below): (i) OM binds to the catalytic domain of the myosin motor at the beginning of the working stroke (step 8) and increases the probability of its attachment to actin (step 9), accelerating the release of Pi (step 10) and preventing the working stroke and the force generation; (ii) OM-ADP motors detach by a process that does not imply ATP binding (step 12); (iii) strongly bound force-generating motors and attached OM-bound motors have the same stiffness, to account for the lack of effect of OM on k_{0} (Fig. 2d, triangles and dashed line) and e_{0} (Fig. 2f); (iv) the AM_{OM-ADP} state generated after Pi release (step 10) undergoes an isomerization (step 11) to a new state (AM_{OM,ADP}) with increased affinity for Pi and thus at [Pi] > 0 (also without added Pi, when contaminating [Pi] is 1 mM) can rebind Pi, release OM and re-enter the force-generating cycle (step 14); in this way, by appropriate selection of the kinetics of steps 11 and 14 (Table 3), the model is able to account for both the OM-dependent reduction in r_{TP} at any [Pi] > 0 (Fig. 1d, continuous line and Fig. 4b), and the Pi-dependent increase in F_{0} in 1 μM OM (Fig. 4d, open circles); (v) the rate limiting step in isometric contraction, that in the conventional cycle is the ADP release (step 5), in the OM cycle is detachment (step 12). Thus k_{+12} is set equal to k_{+5}, to account for the finding that k_{cat} is not affected by [OM] (Fig. 5b triangles) and is reduced by the increase in Pi in the same way in the absence as in the presence of OM (Fig. 5d).

In the presence of OM the probability for an M.ADP.Pi motor to escape the conventional cycle (green pathway in Fig. 6) by binding OM and entering the pink pathway (step 8) is controlled by the second-order equilibrium constant K_{11} which is set at a value 4 · 10^{6} M^{-1} (Table 3) in order to fit the force—[OM] relation of Fig. 1 (continuous line). The kinetics of steps 11, the isomerization that changes Pi affinity of the OM-bound motor, and step 14, in which Pi competes with OM for binding to the attached motor after the isomerization step (step 11), are dictated by the following constraints: (i) without added Pi ([Pi] ~ 1 mM) and in the presence of OM, r_{TP} attains the minimum value ~k_{11} the control with a [OM]_{50} = 0.5 μM (Fig. 1d); (ii) increase in [Pi] reduces β in the same way in the absence and presence of OM (Fig. 4c); (iii) in 1 μM OM the force per motor increases by 50% with the increase of [Pi] in the range 1–10 mM (Fig. 4d). These results are fitted (dashed line) assuming, for step 11 an equilibrium constant (K_{11} = 1, and for step 14, a second-order rate constant for Pi binding (k_{+14}) = 3 · 10^{5} M^{-1} s^{-1} and a second-order rate constant for OM binding (k_{−14}) = 3 · 10^{6} M^{-1} s^{-1} (Table 3).

Discussion

Sarcomere-level mechanics of demembranated fibres from rabbit soleus muscle allow a double mechanism of OM action on the β/ slow MHC isoform to be revealed, which eventually accounts for a Pi-dependent recovery of efficiency of the contractile response of OM-treated fibres.

The first mechanism of action, which confirms the conclusions of recent work in vitro, implies a dose-dependent depression
Table 3 Rate constants for the forward ($k_{+,x}$) and backward ($k_{-,x}$) transitions and corresponding equilibrium constants ($K_x$) for the scheme of Fig. 6.

| Step in the scheme | $k_{+,x}$ | $k_{-,x}$ | $K_x$ | Justification |
|--------------------|----------|----------|-------|---------------|
| A, green-highlighted path in Fig. 6 | | | | |
| (1) | $1 \times 10^6$ M$^{-1}$s$^{-1}$ | $1$ s$^{-1}$ | $1 \times 10^6$ M$^{-1}$ | According to refs. 32,38,62,63 $k_{2}$, according to ref. 38; $k_{20}$ selected to adjust the distribution of detached myosin motors $k_{+,3}$, adjusted to fit the fraction of attached motors in isometric contraction$^{19}$ $k_{+,4}$, from refs. 9,15 (assuming $Q_{10} = 2$) and $K_4$ adjusted to fit the Pi dependence of the rate of force development assuming these steps are 50 times slower than in psoas muscle$^{32}$ $k_{+,5}$, adjusted to fit $k_{cal}$ in isometric contraction in the absence of OM$^{64-66}$ Adjusted to fit the Pi dependence of $k_{cal}$ in the absence of OM$^{37}$ Rapid and irreversible release of hydrolysis products and ATP binding$^{37}$ |
| (2) | $14$ s$^{-1}$ | $9$ s$^{-1}$ | $1.56$ | |
| (3) | $2.2$ s$^{-1}$ | $4.5$ s$^{-1}$ | $0.49$ | |
| (4) | $11$ s$^{-1}$ | $0.9 \times 10^3$ M$^{-1}$s$^{-1}$ | $12.2 \times 10^{-3}$ M | |
| (5) | $1$ s$^{-1}$ | $1 \times 10^3$ M$^{-1}$s$^{-1}$ | $1 \times 10^{-3}$ M | |
| (6) | $0.8$ s$^{-1}$ | $2 \times 10^{-1}$ s$^{-1}$ | $0.4 \times 10^3$ | |
| (7) | $50$ s$^{-1}$ | $- $ | $- $ | |
| B, pink-highlighted path in Fig. 6 | | | | |
| (8) | $400 \times 10^6$ M$^{-1}$s$^{-1}$ | $100$ s$^{-1}$ | $4 \times 10^6$ M$^{-1}$ | Adjusted to have the correct proportion of OM-bound myosin motors $k_{+,9}$ and $k_{-,9}$ adjusted to account for OM-dependent increase of probability of actin attachment by motors ($K_9 > K_3$) and for the constant total number of attached motors in the presence of OM $k_{+,10}$ set to 1.64 $k_{+,4}$, to take into account the effect of OM on the rate of Pi release$^{3}$; $K_{10}$, same value as in step (4) Rapid isomerization step; $K_{10}$ set to 1 to adjust the stoichiometry of the Pi effect Detachment set to fit $k_{cal}$ in the presence of OM Irreversible release of ADP and OM followed by rapid ATP binding Adjusted to fit the kinetics of force development and the Pi dependence of $T_O$ and $e_0$ in the presence of OM |
| (9) | $1.2$ s$^{-1}$ | $1$ s$^{-1}$ | $1.2$ | |
| (10) | $18$ s$^{-1}$ | $1.48 \times 10^3$ M$^{-1}$s$^{-1}$ | $12.2 \times 10^{-3}$ M | |
| (11) | $1000$ s$^{-1}$ | $1000$ s$^{-1}$ | $1$ | |
| (12) | $1.5$ s$^{-1}$ | $2 \times 10^{-1}$ s$^{-1}$ | $1 \times 10^3$ | |
| (13) | $3$ s$^{-1}$ | $- $ | $- $ | |
| (14) | $3 \times 10^3$ M$^{-1}$s$^{-1}$ | $3 \times 10^6$ M$^{-1}$s$^{-1}$ | $1 \times 10^{-3}$ | |

Values for the model in the absence of OM (A, green-highlighted in Fig. 6) and for the branched pathway induced by OM (B, pink-highlighted in Fig. 6). The justification of the kinetic parameters are reported in the last column. Where possible, the parameters are selected according to the relevant information from the literature. The selection of the value of the kinetic parameters concerning OM and OM-Pi actions and the sensitivity of the model fit to the selected value are achieved with an iterative procedure of model simulation by changing one parameter at a time as detailed in “Methods” and in Supplementary Figs. 2 and 3. $x$ represents the step number, as reported in brackets in the first column. For the second-order rate constants, the apparent rate constants are calculated assuming $[MgATP] = 5$ mM and $[ADP] = 20$ μM; [Pi] and [OM] according to the experimental conditions.

Fig. 6 Chemo-mechanical cycle of the myosin motor. The green-highlighted part is the original model$^{37}$; the pink-highlighted part is the branched pathway promoted by OM. Myosin catalytic domain is green for the motors without OM (M) and magenta and brown for the OM-bound motors (MOM) before and after the isomerization step (step 11), respectively. The working stroke in the attached head is indicated by the tilting of the light chain domain (the lever arm, blue) towards the Z-line. The system of linear differential equation for the calculation of state transitions is reported in Supplementary Note 2.
increased total number of attached motors (~twice); at any pCa > 6.8, 1 µM OM acts as a potentiator increasing the sarcomere force, because the increased Ca-sensitivity makes the total number of motors larger than twice the control. This mechanism applies equally well to explain the OM potentiating effects at the partial Ca\(^{2+}\)-activation found in demembranated cardiac myocytes\(^8,29,30\). The force-pCa relation in 1 µM OM is characterized not only by the larger Ca-sensitivity but also by a reduced slope: Hill coefficient \(n\) is reduced by a factor of 2 (from 2.01 to 0.84, Table 2), in agreement with previous work on both demembranated cardiac myocytes and slow-skeletal muscle fibres\(^8,29,30\). The explanation could be related to a negative effect on the cooperativity index of OM-bound motors if the propagation of the activation along the thin filament induced by motor attachment depends on the motor force.

The second mechanism of action of OM, which is revealed in this work, is the recovery from motor force depression with increase in [Pi] in the range 1–10 mM. This finding per se scales back the relevance of previous mechanisms of action of OM defined in the absence of added Pi, since 1–10 mM is the physiological range of [Pi] in working muscle. The molecular basis of this mechanism is implicit in the effect of OM on \(r_{TD}\) also in the absence of added Pi ([Pi] = 1 mM); \(r_{TD}\) is depressed by OM with the same dose dependency as \(T_0\) (Fig. 1 c, d), with [OM] for half-maximum effect 0.5 µM, which noteworthy is in the range (0.1–0.6 µM) of the plasma [OM] in patients under therapeutic treatment\(^3,46\). This consideration suggests further work in which the mechanism revealed here is tested also for the more clinically competent range of [OM].

The kinetic model of Fig. 6 is able to fit both \(T_0\) and \(r_{TD}\)–OM relations (continuous lines in Fig. 1 c and d, respectively) assuming that, after Pi release, the OM-bound motor in the AM\(_{OM}\)-ADP state undergoes an isomerization to an AM\(_{OM}\)-ADP state with higher affinity for Pi favouring an “allosteric” exchange Pi–OM (step 14), which allows the motor to re-enter the force-generating cycle. Noteworthy, it is not possible to simulate the depressant effect of OM on \(r_{TD}\) confining the allosteric competition between OM and Pi to step 10, because the OM-dependent effect on force would not imply any reduction in \(r_{TD}\). Only introducing the pink-highlighted path that contributes to \(T_0\) with a slower process through the isomerization (step 11) and the following OM–Pi exchange (step 14) the time course of \(T_0\) is slowed and the \(r_{TD}\)-Pi relation in 1 µM OM is shifted downward (Fig. 4b). The stoichiometry of the Pi-dependent recovery of the average force per motor in the presence of 1 µM OM (Fig. 4d, open circles) is mainly accounted for by the equilibrium constants of steps 11 (1) and 14 (10\(^{-3}\)). For step 14, assuming a second-order rate constant for Pi binding three orders of magnitude smaller than the second-order rate constant for OM binding, the equilibrium is near the unity when the ratio between [Pi] and [OM] is about 10\(^3\) (Table 3), that is, with the near millimolar physiological concentration of Pi, at micromolar [OM].

The energetics of the combined OM–Pi action on contractility has been defined by determining the effect of OM on the rate of ATP hydrolysis (\(k_{cat}\)) during isometric contraction at saturating Ca\(^{2+}\) and its modulation by Pi. Increase in [OM] up to 2 µM, which decreases \(T_0\) to ½ the control, does not change \(k_{cat}\) (Fig. 5b). This result is specific for a loaded contraction, as in both solution\(^15,35\) and low-load laser trap experiments\(^9\) on β-cardiac myosin the ATPase rate has been found to be reduced in OM with respect to the control.

In either the pink-highlighted or the conventional green-highlighted path under high load the motors accumulate in the ADP-bound state because either OM (pink path) or the high load (green path) limit the probability to go through the working stroke that would accelerate the next step, the conformation
dependent ADP release\textsuperscript{44,47–49}. Consequently, independent of the reason that limits the execution of the working stroke, both step 12 for OM-bound motors and step 5 for OM-free motors are slow (Table 3) and the ATPase rate is limited in the same way independent of the proportion of motors passing through either path. In this way the model can simulate the OM-dependent drop in $T_0$ (dashed line in Fig. 5b) without effect on $k_{act}$ (continuous line).

In the absence of added Pi, OM reduces $T_0$ through a reduction in $F_0$ at constant $k_{act}$, thus it increases the tension cost ($E_1$) of the isometric contraction. One micromolar OM, which halves $F_0$, doubles $E_1$ (Fig. 5e). Noteworthy, this conclusion extends from maximal contractions at saturating [Ca\textsuperscript{2+}] to contractions at sub-saturating [Ca\textsuperscript{2+}] because reduction in [Ca\textsuperscript{2+}] reduces the total number of attached motors (Fig. 3b) without affecting $F_0$ (Fig. 3c).

A direct test of Pi effect on OM-bound motors at pCa $\sim$ 7, where OM exerts its potentiating action via the increase in Ca\textsuperscript{2+} sensitivity (Fig. 3d), would be quite important, considering that the internal [Ca\textsuperscript{2+}] in the heart systole ranges 0.2–0.6 mM\textsuperscript{50,51}. However, the sarcoplasmic reticulum of male New Zealand white rabbits at either the physiological range (1–10 mM) recovers from the inhibitory action of OM (which reduces $T_0$, $E_0$ at constant $k_{act}$) and the half-sarcomere compliance analysis would be complicated by the significant contribution of the additional elasticity in parallel with the motor array (see “Methods”, Fig. 7b).

In this respect it must be noted also that the nature and the effects of the additional parallel elasticity are likely different in the slow-skeletal muscle and in the cardiac muscle, which have different isoforms of the accessory and cytoskeleton proteins like My-BPC and titin. Consequently, the conclusions from soleus muscle could not be directly translated to cardiac muscle as it is instead complicated by the significant contribution of the additional elasticity in parallel with the motor array (see “Methods”, Fig. 7b). In the absence of added Pi, OM reduces $T_0$ through a reduction in $F_0$ at constant $k_{act}$, thus it increases the tension cost ($E_1$) of the isometric contraction. One micromolar OM, which halves $F_0$, doubles $E_1$ (Fig. 5e). Noteworthy, this conclusion extends from maximal contractions at saturating [Ca\textsuperscript{2+}] to contractions at sub-saturating [Ca\textsuperscript{2+}] because reduction in [Ca\textsuperscript{2+}] reduces the total number of attached motors (Fig. 3b) without affecting $F_0$ (Fig. 3c).

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of $T_g > 50$ kPa and in the presence of OM are reported in Table 1. The value of $C_i$ in the presence of OM (15.3 nm MPa$^{-1}$) does not differ significantly from the control value (15.2 nm MPa$^{-1}$, $P > 0.9$, Student's t test), indicating that also in this case the dependence of $T_g$ on [Ca$^{2+}$] is explained by the Ca$^{2+}$-modulation of the number of attached motors without change in motor strain.

In OM the $Y_o - T_g$ relation (open circles in Fig. 7c) does not show deviation from linearity for the same low forces at which the control relation shows a downward shift. This apparent contradiction is explained by considering the effect of OM that reduces the force per motor but increases the Ca-sensitivity for motor attachment, so that at the same low values of $T_g$ in the presence of OM, the number of attached motors and thus their cumulative stiffness are higher than in control and still in the range of values that mask the contribution of the parallel elastic element. A quantitative evaluation of this interpretation is obtained in Fig. 7d by plotting $Y_o$ versus the stiffness of the array of motors $e_o$ at each $pC$ (data from Fig. 3b) for both control (filled circles) and OM (open circles). It can be seen that both control and OM relations are almost linear in the same range of $e_o$, where the stiffness of attached motors is high enough to mask the contribution of the parallel elastic element, and that the control relation deviates from linearity, uncovering the presence of a parallel elastic element, for the small $e_o$ values that are not attained by the OM relation.

**ATPase measurements.** Fibre bundles prepared in Florence lab as described above were transported to Amsterdam in ice-cold relaxing solution. ATPase activity was measured at saturating [Ca$^{2+}$] at different [Ca$^{2+}$] measured photometrically by enzymatic coupling of the regeneration of ATP to the oxidation of NADH present in the bathing solution$^{36,60,61}$ both in control and in the presence of OM (1 and 2 μM). NADH breakdown was monitored via the absorption of near UV light at 340 nm. Briefly, the set up consisted of two anodized aluminium troughs (volume 90 μl each) containing relaxing pre-activating solution and a measuring chamber (volume about 30 μl) containing activating solution. The solution was continuously stirred via a membrane at the base of the chamber. UV light passed through the chamber beneath the fibre, and the transmitted light was monitored by two UV-enhanced photodiodes at 340 and 400 nm. The photodiode at 400 nm provided a reference signal, independent of NADH concentration. A syringe, controlled by a stepper motor, was used to add 0.05 μl of 10 mM ADP at the end of each recording to calibrate the absorbance signal. Force during the enzymatic assay was measured by means of a strain gauge transducer (AE801 Sensonor, Horten, Norway). The force and the absorbance signals were filtered at 1 kHz and 2.5 Hz, respectively. The ATPase rate was measured as the slope over time of the absorbance signal.

**Procedure for the selection of the parameters of the kinetic model.** The procedure followed to assign a specific value to the kinetic parameters concerning the effects of OM and Pi on the force ($T_g$), rate of force development ($r_{TD}$), stiffness of the motor array ($e_o$) and ATPase rate ($R_p$) (eight relations altogether) is based on an iterative routine in which the values of the rate constants (or equilibrium constants) controlling the kinetics of the relevant steps (8–12 and 14) are changed one at a time. For each value of the rate/equilibrium constant ($k$) the weighted mean of residual errors for each relation is calculated according to the expression:

$$R_w(k) = \frac{1}{N} \sum_{i=1}^{N} \left( \frac{y_i - x_{mod}(k)}{\sigma_i} \right)^2,$$

where $R_w(k)$ is the mean sum of residuals (weighted for the squared value of the experimental point to allow homogeneous comparison among the eight different relations), $x_i$ is the experimental point value, $x_{mod}(k)$ is the corresponding simulated value for a given $k$, and $N$ is the number of points in each relation. An example of the selection procedure for a given rate constant ($k_{12}$) in relation to its effect on each of the eight relevant relations is given in Supplementary Fig. 2, where in the left column six simulations obtained for different values of $k_{12}$ (each identified by a different colour) of four of the eight relations are superimposed on the experimental relations (open symbols) and, in the right column, the weighted mean of residuals ($R_w(k)$) are plotted versus the value of $k_{12}$ assumed in each simulation (identified by the same colour as in the left column). As shown by the plots in the right column of Supplementary Fig. 2, the sensitivity of the test in showing a minimum mean residual and its dependence on the value of the rate/equilibrium constant varies depending on the relation. For this, the criterium of selection of the best value for any given rate/equilibrium constant is based on the weighted mean of the residuals averaged over the eight relations (global mean, $\langle R_w(k) \rangle$):

$$\langle R_w(k) \rangle = \frac{1}{8} \sum_{i=1}^{8} R_w(k).$$

In Supplementary Fig. 3 the values of $\langle R_w(k) \rangle$ are plotted versus the rate/equilibrium constant under consideration. The value selected with this global fitting for each rate/equilibrium constant, identified by the abscissa of the filled symbol, is reported in Table 3.

**Solutions.** The composition of the solutions, similar for the mechanical and the ATPase measurements (MgATP, 5 mM; free Mg$^{2+}$, 1.2 mM) is reported in Supplementary Table 1. The activating solution at a given pCa (range 7.5–4.5) was obtained by mixing relaxing and activating solutions. OM (CK-1827452 from Selleckchem, USA) was dissolved in DMSO (Sigma D-5879) (stock solution, 17.5 mM). Different OM concentrations in the final solutions (0.1–10 μM range) were obtained for partial dilution starting from the stock solution. Four percent dextran was added to all solutions for the mechanical experiments.

**Statistical analysis.** All data were analyzed using dedicated programs written in LabVIEW (National Instruments) and Microsoft Excel and Origin 2018 (OriginLab Corp., Northampton, MA, USA) software. Error bars on mean data points are ±SEM.

For mean data in the figures and tables, the number of fibres is reported in the legends and the number of observations, which may vary for each data point, can be recovered from the Source Data files.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. The source data for Figs. 1, 2, 3, 4, 5, 7, Supplementary Figs. 1, 2 and 3 and for Tables 1, 2 and Supplementary Table 1 are provided as a Source Data file. All remaining data will be available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
V.L., M.L., G.P., M.C., C.O. and G.S. designed the research. S.G., M.C. and I.P. performed the experiments and analysed the data. V.L., M.L. and G.P. wrote the paper or revised it critically for important intellectual content. All authors participated in discussions on this work and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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