

## RESEARCH PAPER

# The novel cardiac myosin activator omecamtiv mecarbil increases the calcium sensitivity of force production in isolated cardiomyocytes and skeletal muscle fibres of the rat

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## BACKGROUND AND PURPOSE

Omecamtiv mecarbil (OM) is a novel cardiac myosin activator drug for inotropic support in systolic heart failure. Here we have assessed the concentration-dependent mechanical effects of OM in permeabilized cardiomyocyte-sized preparations and single skeletal muscle fibres of Wistar-Kyoto rats under isometric conditions.

## EXPERIMENTAL APPROACHES

Ca<sup>2+</sup>-dependent active force production ( $F_{\text{active}}$ ), its Ca<sup>2+</sup> sensitivity ( $pCa_{50}$ ), the kinetic characteristics of Ca<sup>2+</sup>-regulated activation and relaxation, and Ca<sup>2+</sup>-independent passive force ( $F_{\text{passive}}$ ) were monitored in Triton X-100-skinned preparations with and without OM (3nM–10  $\mu$ M).

## KEY RESULTS

In permeabilized cardiomyocytes, OM increased the Ca<sup>2+</sup> sensitivity of force production ( $\Delta pCa_{50}$ : 0.11 or 0.34 at 0.1 or 1  $\mu$ M respectively). The concentration–response relationship of the Ca<sup>2+</sup> sensitization was bell-shaped, with maximal effects at 0.3–1  $\mu$ M OM ( $EC_{50}$ :  $0.08 \pm 0.01$   $\mu$ M). The kinetics of force development and relaxation slowed progressively with increasing OM concentration. Moreover, OM increased  $F_{\text{passive}}$  in the cardiomyocytes with an apparent  $EC_{50}$  value of  $0.26 \pm 0.11$   $\mu$ M. OM-evoked effects in the diaphragm muscle fibres with intrinsically slow kinetics were largely similar to those in cardiomyocytes, while they were less apparent in muscle fibres with fast kinetics.

## CONCLUSIONS AND IMPLICATIONS

OM acted as a Ca<sup>2+</sup>-sensitizing agent with a downstream mechanism of action in both cardiomyocytes and diaphragm muscle fibres. The mechanism of action of OM is connected to slowed activation–relaxation kinetics and at higher OM concentrations increased  $F_{\text{passive}}$  production.

## Abbreviations

cTnC, cardiac troponin C;  $F_{\text{active}}$ , active force;  $F_{\text{max}}$ , maximal force level;  $F_{\text{passive}}$ , passive force;  $F_{\text{total}}$ , total force level; HF, heart failure;  $k_{\text{tr}}$ , rate constant of force redevelopment at submaximal  $[\text{Ca}^{2+}]$ ;  $k_{\text{tr,max}}$ , rate constant of force redevelopment at pCa 4.75; LV, left ventricle; MHC, myosin heavy chain;  $n_{\text{Hill}}$ , Hill coefficient; OM, omecamtiv mecarbil; pCa,  $-\log$  of calcium ion concentration; pCa<sub>50</sub>,  $-\log$  of calcium ion concentration at half-maximal isometric force production;  $P_i$ , inorganic phosphate;  $t_{\text{relax}}$ , relaxation time

## Introduction

While heart failure (HF) specialists continue to wait for better opportunity for the characterization of patient populations or clinical conditions so as to optimize the outcome of therapy with conventional positive inotropes (McMurray *et al.*, 2012), the search for new cardiotoxic agents remains ongoing (Nagy *et al.*, 2014). In this aspect, pharmacological targeting of the myocardial contractile protein machinery is an appealing concept for the treatment of the weakened cardiac contractions during acute and decompensated HF (Endoh, 2008). Direct activation of the cardiac myosin heads has recently been introduced as a novel approach for the treatment of systolic HF. Hence, the cardiac myosin activator omecamtiv mecarbil (OM) has been proved to be effective in animal models *in vitro* and *in vivo* (Malik *et al.*, 2011). Indeed, emerging clinical experience lends support to this therapy (Cleland *et al.*, 2011).

Considering the interaction between  $\text{Ca}^{2+}$  and the cardiac troponin C (cTnC) as a reference point within the contractile activation process, inotropic interventions can be classified as those with (i) upstream mechanisms (increasing the amplitude of the intracellular  $\text{Ca}^{2+}$  transient); (ii) central mechanisms (promoting the interaction between  $\text{Ca}^{2+}$  and cTnC); and (iii) downstream mechanisms (directly modulating the actin–myosin interactions; Endoh, 2001). The latter two actions are conventionally lumped together as  $\text{Ca}^{2+}$ -sensitizing positive inotropy (Vannier *et al.*, 1997; Szilagyi *et al.*, 2004). By accelerating the inorganic phosphate ( $P_i$ ) release from the cardiac myosin heads, myosin activation implicates a molecular mechanism, similar to those with a downstream inotropic action. Therefore, it is not surprising that OM increases the  $\text{Ca}^{2+}$  sensitivity of the myosin ATPase activity (Malik *et al.*, 2011), but an OM-dependent  $\text{Ca}^{2+}$ -sensitizing effect on isometric force production has not been documented to date.

Downstream  $\text{Ca}^{2+}$  sensitizer agents have been found to increase the  $\text{Ca}^{2+}$ -independent passive force ( $F_{\text{passive}}$ ) of the cardiomyocytes, and thereby to impair the diastolic function of the heart. Accordingly, prolonged relaxation has been observed in the presence of EMD-57033, CGP-48506 and ORG-30029 (Miller and Steele, 1990; Brixius *et al.*, 2000; Papp *et al.*, 2004; Choi *et al.*, 2010). The relative proximity of the OM docking site on the myosin heavy chain (MHC) to the binding site of EMD-57033 implies potential similarities between their mechanisms of action (Radke *et al.*, 2014) but a potential effect of OM on the  $F_{\text{passive}}$  remained unclear.

The effect of OM is considered to be selective for the myocardium, though its target molecule, the cardiac myosin  $\beta$ -heavy chain ( $\beta$ -MHC), is also expressed in certain skeletal muscle fibres (Brenner *et al.*, 2012). OM may therefore influ-

ence the contractility of the diaphragm and other slow-twitch muscle fibres which also co-express  $\beta$ -MHC, but these potential effects have not been addressed experimentally so far.

The aims of our present investigations were therefore to identify potential OM-dependent  $\text{Ca}^{2+}$ -sensitizing effects in cardiomyocytes and in diaphragm muscle fibres, and to determine how OM influences the mechanics and kinetics of their activation–relaxation cycles. These results will hopefully shed new light on the mechanism of action of OM and its tissue selectivity, and hence provide information concerning its clinical applicability.

## Methods

### *Muscle specimens and the animal welfare and ethical statement*

All animal care and experimental procedures involved in this work conformed strictly to Directive 2010/63/EU of the European Parliament and were approved by the Ethical Committee of the University of Debrecen (Ethical Statement number: 1/2013/DE MÁB). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 11 animals were used in the experiments described here.

Investigations were carried out on male 8–15-week-old Wistar-Kyoto rats, weighing 250–500 g (Toxi-Coop Toxicological Research Centre, Dunakeszi, Hungary). The animals were fed a standard chow and drank tap water *ad libitum*. The rats were housed in groups of two to three per polycarbonate cages in a room controlled thermostatically at  $20 \pm 2^\circ\text{C}$  with a target relative humidity of  $55 \pm 15\%$ . The room was artificially illuminated with fluorescent lights from 6:00 a.m. to 8:00 p.m. All procedures were as humane as possible to minimize the distress of the animals. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Release, Garbsen, Germany;  $150 \text{ mg}\cdot\text{kg}^{-1}$ ). The heart and the diaphragm were quickly excised and the left ventricle (LV) was dissected in cold isolating solution ( $\text{MgCl}_2$ : 1 mM, KCl: 100 mM, EGTA: 2 mM, ATP: 4 mM, imidazole: 10 mM; pH 7.0). Diaphragm and LV tissue samples were stored at  $-80^\circ\text{C}$ .

### *Mechanical measurements on permeabilized myocyte-sized preparations*

Deep-frozen LV and diaphragm tissue samples were mechanically disrupted in isolating solution using a tissue homogenizer at  $4^\circ\text{C}$ , and thereafter subjected to chemical

permeabilization with 0.5% Triton X-100 detergent for 5 min, as described previously (Papp *et al.*, 2004). The Triton X-100 eliminated all membrane structures, allowing characterization of the mechanical properties of single myocytes under standardized conditions with the avoidance of potential disturbing factors present *in vivo*.

Isolated and permeabilized single cardiomyocytes or skeletal muscle fibres were kept in relaxing solution (BES: 10 mM; KCl: 37.11 mM; MgCl<sub>2</sub>: 6.41 mM; EGTA: 7 mM; ATP: 6.94 mM; creatine phosphate: 15 mM; pH 7.2) and were attached with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD, USA) to two stainless insect needles, connected to a very sensitive force transducer (Sensonor, Horten, Norway) and an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). After adjustment of the sarcomere length to 2.3 μm, the contractile machinery was activated by transferring the preparation from the relaxing to the activating solution (the same components apart from containing Ca<sup>2+</sup>-EGTA instead of EGTA). Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]) were indicated as -log of calcium ion concentration (pCa) values, calculated by -lg[Ca<sup>2+</sup>]. The compositions of the activating (pCa 4.75) and relaxing solution (pCa 9.0) were calculated by using a previously reported approximation (Fabiato and Fabiato, 1979). All solutions contained protease inhibitors: phenylmethylsulfonyl fluoride: 0.5 mM, leupeptin: 40 μM and E-64: 10 μM. All chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

The Ca<sup>2+</sup>-induced contractions of the preparations were recorded with a custom-built LABVIEW Data Acquisition platform and analysed with LabVIEW analysing software (Myo; National Instruments, Austin, TX, USA). When the Ca<sup>2+</sup>-activated force production reached its maximal, a quick release-retch manoeuvre (30 ms) was applied in the activating solution. As a result of this intervention, force first dropped to zero and then started to redevelop allowing the determination of the total force ( $F_{\text{total}}$ ). The Ca<sup>2+</sup>-independent  $F_{\text{passive}}$  was approximated by shortening of the preparations to 80% of the original lengths in relaxing solution for 8 s. The active isometric force ( $F_{\text{active}}$ ) was calculated as the difference between  $F_{\text{total}}$  and  $F_{\text{passive}}$ . Force values were normalized for the cross-sectional area determined from the width and height of the myocytes, approximating their dimensions with elliptical geometry. To test the stability of the preparations, reference activations (pCa 4.75) were performed at the end of each experiment. Preparations were discarded when maximal force level ( $F_{\text{max}}$ ) declined to less than 80% of that recorded at the beginning of each experiment.

### Analysis of the MHC isoform composition

The MHC isoform composition of the rat diaphragm and LV was analysed, as described elsewhere, using SDS-PAGE (Talmadge and Roy, 1993). Deep-frozen muscle samples were homogenized and then boiled in sample buffer for 2 min. The adjusted final protein concentration was 0.25 mg·mL<sup>-1</sup>. The stacking gel contained 30% glycerol, 4% acrylamide-bis, 70 mM Tris, 4 mM EDTA and 0.4% SDS. The separating gel consisted of 30% glycerol, 6% acrylamide-bis, 0.2 mM Tris, 4 mM EDTA and 0.4% SDS. The polymerization of the gels was induced by 0.05% tetramethylethylenediamine and 0.1% ammonium persulfate. Separate upper and lower buffers were used. The upper running buffer was composed of 0.1 mM

Tris, 150 mM glycine and 0.1% SDS and the lower running gel of 50 mM Tris, 75 mM glycine and 0.05% SDS. The running conditions were 70 V (constant voltage) for 24 h at 4°C. The gels were stained with Coomassie Blue.

### Data analysis

Isometric force values ( $F$ ) at a given [Ca<sup>2+</sup>] (pCa 5.4–9.0), normalized to  $F_{\text{max}}$  were fitted to a modified Hill equation in Origin 6.0 (Microcal Software, Northampton, MA, USA) or GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, CA, USA):

$$F = F_{\text{max}} * [\text{Ca}^{2+}]^{n_{\text{hill}}} / (\text{Ca}_{50}^{n_{\text{hill}}} + [\text{Ca}^{2+}]^{n_{\text{hill}}} + F_{\text{passive}})$$

The pCa value for the half-maximal contraction indicated by  $\text{Ca}_{50}$  or  $\text{pCa}_{50}$  defines the Ca<sup>2+</sup> sensitivity of force generation of the contractile machinery, while the steepness of the Ca<sup>2+</sup> sensitivity curve describing the myofibrillar co-operativity was expressed as a coefficient ( $n_{\text{hill}}$ ).

The force redevelopment following the release-retch manoeuvre was fitted to a single exponential to evaluate the rate constant of force redevelopment ( $k_{\text{tr}}$ ) indicating the intrinsic kinetics of the actin–myosin cross-bridges. The half-time of activation ( $t_{1/2}$  of activation) was determined by estimating the duration for half-maximal contraction after the preparations were placed in activating solution. The transfer of the preparations into the relaxing solution was associated with a short artefact on the force records. Relaxation time ( $t_{\text{relax}}$ ) was defined as the difference in time between this artefact and the moment when force returned to baseline level. Statistical significance, accepted at  $P < 0.05$ , was calculated by ANOVA (repeated measures) followed by Dunnett's two-tailed test or by paired Student *t*-test, as appropriate. Statistical analyses were carried out by GraphPad Prism. Values are given as means ± SEM.

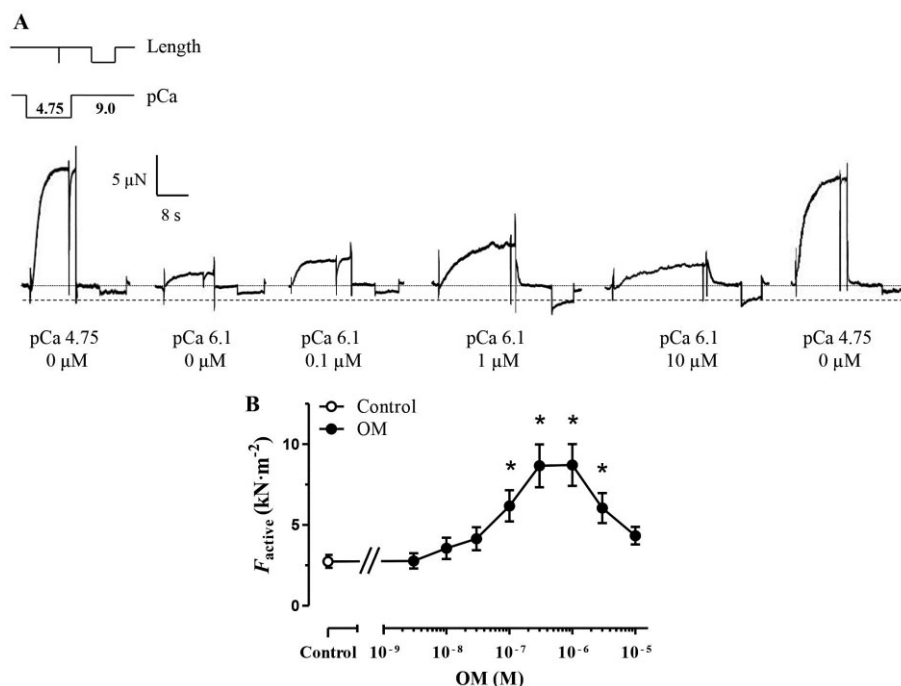
### Materials

OM was purchased from AdooQ BioScience (Irvine, CA, USA). Stock solutions with a final OM concentration of 10 mM were prepared in DMSO as solvent and stored at 4°C. Appropriate volumes of the concentrated stock solutions were dissolved in activating and relaxing solutions to obtain test solutions containing OM in the concentration range between 3 nM and 10 μM. The final concentration of DMSO never exceeded 0.1%.

## Results

### The Ca<sup>2+</sup>-sensitizing effects of OM in isolated cardiomyocytes

The Ca<sup>2+</sup>-sensitizing effect of OM was first investigated in LV myocyte-sized preparations. As illustrated by typical force recordings, the amplitudes of  $F_{\text{active}}$  production increased with OM concentrations upon repeated activations in test solutions at a submaximal [Ca<sup>2+</sup>] (pCa 6.1). The maximal increase in isometric force was reached in the presence of 1 μM OM. Contractile force was not enhanced further by higher OM concentrations, and it rather tended to decline towards the amplitude in OM-free conditions. This behaviour could not be attributed to preparation run-down as verified by the



**Figure 1**

The  $\text{Ca}^{2+}$ -sensitizing effect of OM in permeabilized cardiomyocytes of the rat. (A) Force recordings illustrate  $\text{Ca}^{2+}$ -induced contractions with increasing amplitudes at a submaximal  $[\text{Ca}^{2+}]$  (pCa 6.1), due to the  $\text{Ca}^{2+}$ -sensitizing effect of increasing OM concentrations in a permeabilized cardiomyocyte under isometric conditions. During repeated activation–relaxation cycles, myocyte-sized preparations were first equilibrated in relaxing solution with an OM concentration between 3 nM and 10  $\mu\text{M}$  for 5 min and subsequently activated in the presence of the same OM concentration at submaximal  $[\text{Ca}^{2+}]$ . Control maximal activations (pCa 4.75) in the absence of OM were performed to determine  $F_{\text{max}}$  and preparation run-down. Length and  $[\text{Ca}^{2+}]$  changes are schematically given above the first activation–relaxation cycle. Cardiomyocyte transfers between relaxing (pCa 9.0) and activating solutions (pCa 4.75 or pCa 6.1) were accompanied by brief vertical force artefacts. pCa values and OM concentrations are indicated below the individual force records. The horizontal dotted line depicts the force baseline in relaxing solution and the dashed line the increased  $F_{\text{passive}}$  level seen in the presence of 1  $\mu\text{M}$  or 10  $\mu\text{M}$  OM. (B) OM exhibited a bell-shaped concentration–response relationship on the isometric force production ( $n = 8$  cardiomyocytes from four different animals). Increasing concentrations of OM was applied in the same myocyte-sized preparations. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.

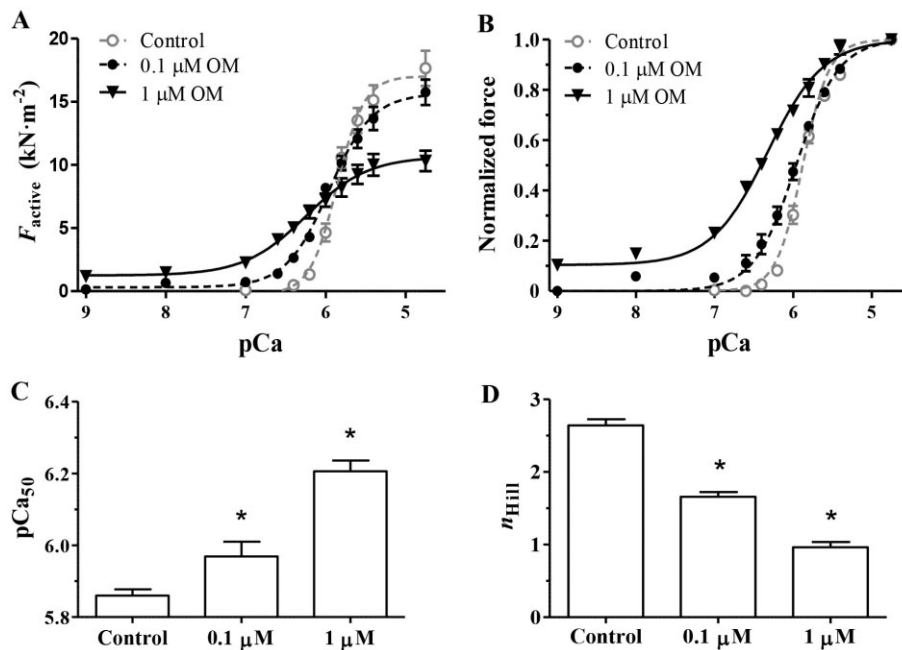
bracketing of test activations under OM-free conditions at saturating  $[\text{Ca}^{2+}]$  (resulting in comparable force amplitudes at the beginning and at the end of our protocols; Figure 1A). The OM-evoked increase in  $F_{\text{active}}$  at pCa 6.1 was significant in the OM concentration range between 0.1  $\mu\text{M}$  and 3  $\mu\text{M}$ . The concentration–response relationship of OM was bell-shaped, with the highest levels of  $\text{Ca}^{2+}$  sensitization between 0.3  $\mu\text{M}$  and 1  $\mu\text{M}$  OM ( $F_{\text{active}}$  in OM-free medium:  $2.74 \pm 0.40 \text{ kN}\cdot\text{m}^{-2}$  vs.  $8.66 \pm 1.32 \text{ kN}\cdot\text{m}^{-2}$  and  $8.71 \pm 1.29 \text{ kN}\cdot\text{m}^{-2}$  at 0.3 and 1  $\mu\text{M}$  OM, respectively;  $P < 0.001$  vs. control; Figure 1B), while no  $\text{Ca}^{2+}$ -sensitizing effect was apparently exerted at 10  $\mu\text{M}$  OM. The concentration–response relationship was fitted to a sigmoid function in the OM concentration range between 3 nM and 1  $\mu\text{M}$  resulting in an  $\text{EC}_{50}$  of  $0.08 \pm 0.01 \mu\text{M}$ .

Thereafter, the effects of OM on the pCa–force relationship were tested in detail by exposing myocyte-sized preparations to activating solutions with maximal (pCa 4.75) or submaximal  $[\text{Ca}^{2+}]$  (i.e. between pCa 9.0 and pCa 5.4) in the presence of a low (0.1  $\mu\text{M}$ ) or an intermediate (1  $\mu\text{M}$ ) concentration of OM. 0.1  $\mu\text{M}$  OM did not affect the force production relative to the OM-free conditions either at the maximal (i.e. at pCa 4.75) or at the lowest applied  $[\text{Ca}^{2+}]$  (pCa 9), whereas it promoted force production at intermediate levels of acti-

vation (between pCa 6.6 and pCa 5.6) and hence shifted the pCa–force relationship to the left. In the presence of 1  $\mu\text{M}$  OM, the force augmentation was pronounced at low–intermediate  $[\text{Ca}^{2+}]$  levels (i.e. between pCa 9.0 and pCa 6.0), albeit decreases in  $F_{\text{max}}$  together with force reductions at high–intermediate  $\text{Ca}^{2+}$  concentrations were also observed (i.e. between pCa 5.6 and pCa 5.4). Means of force amplitudes are expressed in absolute terms ( $\text{kN}\cdot\text{m}^{-2}$ ; Figure 2A) and normalized terms (Figure 2B). The leftward shift on the normalized pCa–force relationship indicated increased  $\text{Ca}^{2+}$  sensitivity of force production (pCa<sub>50</sub> in the OM-free medium:  $5.86 \pm 0.02$  vs.  $5.97 \pm 0.02$  and  $6.20 \pm 0.03$  at 0.1 and 1  $\mu\text{M}$  OM, respectively;  $P < 0.001$  in both groups vs. the control; Figure 2C). 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  OM decreased  $n_{\text{Hill}}$  of the normalized pCa–force relationships ( $n_{\text{Hill}}$  in OM-free medium:  $2.64 \pm 0.09$  vs.  $1.66 \pm 0.06$  and  $0.97 \pm 0.07$  at 0.1 and 1  $\mu\text{M}$  OM, respectively;  $P < 0.001$  in both groups vs. the control; Figure 2D).

#### *Effects of OM on the mechanics and kinetics of activation–relaxation cycles in isolated cardiomyocytes*

To obtain a more detailed picture of how OM affected the kinetics of  $\text{Ca}^{2+}$ -dependent activations and relaxations, iso-



**Figure 2**

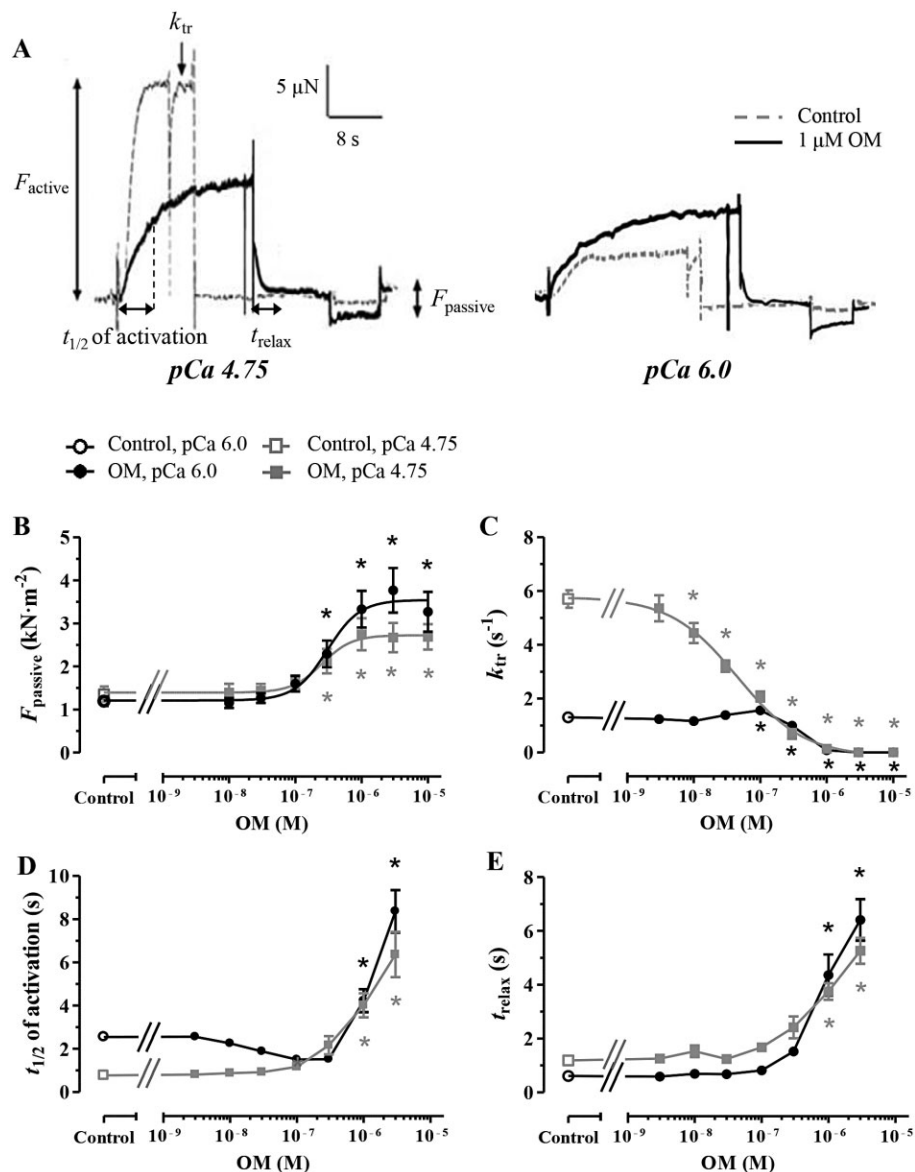
The  $\text{Ca}^{2+}$ -sensitizing effect of OM in permeabilized cardiomyocytes was quantified by pCa–force relationships ( $[\text{Ca}^{2+}]$  between pCa 9 and pCa 4.75) in the absence and the presence of 0.1  $\mu\text{M}$  or 1  $\mu\text{M}$  OM. (A, B) Means of force amplitudes are expressed in absolute ( $\text{kN}\cdot\text{m}^{-2}$ ) and normalized terms. (C) OM-evoked  $\text{Ca}^{2+}$  sensitization and the resultant leftward shifts in the pCa–force relations are illustrated by the means of their midpoints ( $\text{pCa}_{50}$ ) in the absence and presence of 0.1 or 1  $\mu\text{M}$  OM. (D) 0.1 or 1  $\mu\text{M}$  OM decreased the co-operativity of  $\text{Ca}^{2+}$ -regulated force production ( $n_{\text{Hill}}$ ) in permeabilized cardiomyocytes ( $n = 8$  cardiomyocytes from three different animals for each group). Each curve was generated with increasing concentrations of OM in the same cells. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.

metric force measurements were carried out at saturating (pCa 4.75) and submaximal (pCa 6.0)  $[\text{Ca}^{2+}]$  using a wide range of OM concentrations (between 3 nM and 10  $\mu\text{M}$ )  $F_{\text{active}}$ ,  $F_{\text{passive}}$ ,  $k_{\text{tr}}$ ,  $t_{1/2}$  of activation and  $t_{\text{relax}}$  were determined (Figure 3A). OM decreased  $F_{\text{active}}$  in a monotonous fashion at pCa 4.75, significantly at 1  $\mu\text{M}$  OM concentration or higher ( $F_{\text{max}}$  in OM-free medium:  $14.75 \pm 0.70 \text{ kN}\cdot\text{m}^{-2}$  and at 1  $\mu\text{M}$  OM concentration:  $9.42 \pm 0.97 \text{ kN}\cdot\text{m}^{-2}$ ,  $P < 0.001$  vs. the control); however, the concentration–response relationship of OM was bell-shaped at pCa 6.0 due to  $\text{Ca}^{2+}$  sensitization (Supporting Information Fig. S1a) similarly to that shown in Figure 1B. OM increased  $F_{\text{passive}}$  at 0.3  $\mu\text{M}$  and higher concentration at pCa 4.75 and pCa 6.0 ( $\text{EC}_{50}$ :  $0.30 \pm 0.10 \mu\text{M}$  and  $0.26 \pm 0.11 \mu\text{M}$  at pCa 4.75 and pCa 6.0, respectively; Figure 3B). At pCa 4.75, OM decreased  $k_{\text{tr}}$  at 10 nM and higher concentration ( $k_{\text{tr}}$  in OM-free medium:  $5.70 \pm 0.33 \text{ s}^{-1}$  and  $4.44 \pm 0.37 \text{ s}^{-1}$  at 10 nM OM;  $P < 0.01$  vs. the control;  $\text{EC}_{50}$ :  $0.05 \pm 0.01 \mu\text{M}$ ). Interestingly, OM-dependent changes of  $k_{\text{tr}}$  were biphasic at pCa 6.0:  $k_{\text{tr}}$  increased in the presence of 0.1  $\mu\text{M}$  OM from the drug-free control of  $1.31 \pm 0.08 \text{ s}^{-1}$  to  $1.57 \pm 0.08 \text{ s}^{-1}$  ( $P < 0.05$  vs. the control), albeit  $k_{\text{tr}}$  decreased in the presence of 0.3  $\mu\text{M}$  and higher OM concentrations (to  $1.00 \pm 0.11 \text{ s}^{-1}$  at 0.3  $\mu\text{M}$  OM;  $P < 0.01$  vs. the control; Figure 3C). Similar kinetic features were observed on the prolongation of  $t_{1/2}$  of activation. 1  $\mu\text{M}$  and higher concentrations of OM increased significantly the  $t_{1/2}$  of activation at pCa 4.75 ( $t_{1/2}$  in OM-free conditions:

$0.77 \pm 0.12 \text{ s}$  and  $4.02 \pm 0.56 \text{ s}$  at 1  $\mu\text{M}$  OM,  $P < 0.001$  vs. the control) and at pCa 6.0 ( $t_{1/2}$  in OM-free conditions:  $2.55 \pm 0.19 \text{ s}$  and  $4.23 \pm 0.53 \text{ s}$  at 1  $\mu\text{M}$  OM,  $P < 0.05$  vs. the control). Nevertheless,  $t_{1/2}$  of activation tended to decrease at pCa 6.0 in response to intermediate concentrations of OM (30 nM–0.3  $\mu\text{M}$ ), without reaching statistical significance (Figure 3D). The relaxation was slowed following  $\text{Ca}^{2+}$ -removal from the preparations at 1  $\mu\text{M}$  and higher OM concentrations at pCa 4.75 ( $t_{\text{relax}}$  in the OM-free medium:  $1.19 \pm 0.10 \text{ s}$  and  $3.75 \pm 0.31 \text{ s}$  at 1  $\mu\text{M}$  OM;  $P < 0.001$  vs. control) and at pCa 6.0 ( $t_{\text{relax}}$  in the OM-free medium:  $0.61 \pm 0.06 \text{ s}$  and  $4.36 \pm 0.77 \text{ s}$  at 1  $\mu\text{M}$  OM;  $P < 0.001$  vs. control; Figure 3E). The  $t_{1/2}$  of activation and  $t_{\text{relax}}$  could not be determined at higher than 3  $\mu\text{M}$  OM concentrations because of low isometric force production.

### *The $\text{Ca}^{2+}$ -sensitizing effects of OM in isolated skeletal muscle fibres with slow intrinsic kinetics*

To assess any  $\text{Ca}^{2+}$ -sensitizing effects of OM in skeletal muscles, measurements similar to those carried out in cardiomyocytes were repeated on isolated muscle fibres derived from the rat diaphragm. The force records depicted in Figure 4A demonstrated that the contractile force of the rat diaphragm was enhanced in response to increasing OM concentrations at a submaximal level of activation (pCa 6.0), as observed in cardiomyocytes. A complicating factor in dia-

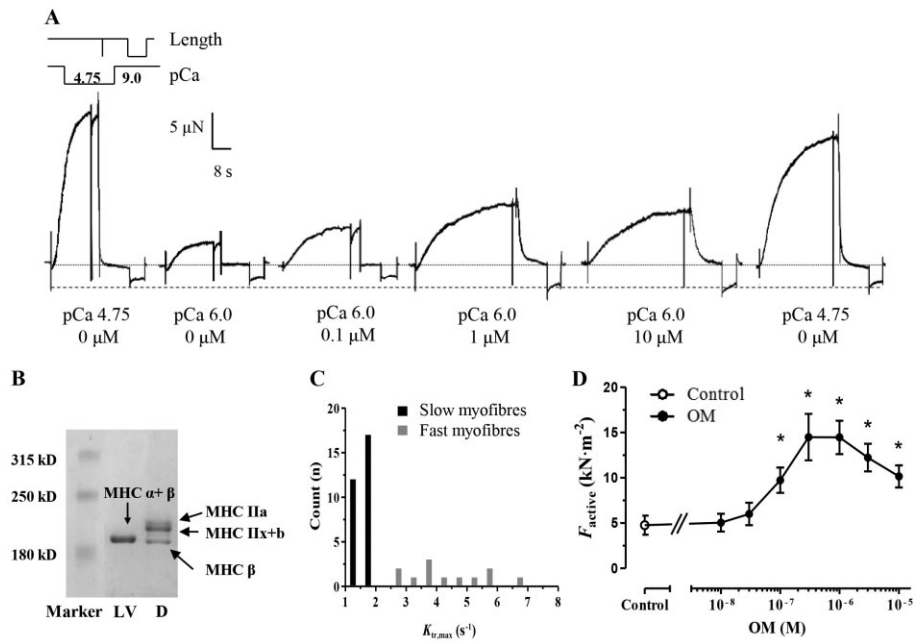


**Figure 3**

The concentration dependence of OM-induced mechanical and kinetic changes in permeabilized cardiomyocytes at saturating (pCa 4.75) and submaximal (pCa 6.0)  $[\text{Ca}^{2+}]$ . (A) Representative force records at pCa 4.75 (left panel) or pCa 6.0 (right panel) illustrated the effects of 1  $\mu\text{M}$  OM on  $F_{\text{active}}$ ,  $F_{\text{passive}}$ ,  $k_{\text{tr}}$ ,  $t_{1/2}$  of activation and  $t_{\text{relax}}$ , together with controls in the absence of OM. (B) OM increased  $F_{\text{passive}}$  similarly at pCa 4.75 and 6.0. (C) The  $k_{\text{tr}}$  decreased at pCa 4.75 with OM concentration; however, it showed a biphasic OM concentration dependence at pCa 6.0. (D, E)  $t_{1/2}$  of activation and  $t_{\text{relax}}$  were prolonged after OM treatment at saturating and submaximal  $[\text{Ca}^{2+}]$ .  $n = 8$  muscle fibres at pCa 4.75 and 7–11 muscle fibres at pCa 6.0, from 3–4 different animals respectively. Increasing concentrations of OM was applied in the same myocyte-sized preparations. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.

phragm muscle preparations is the composition of muscle fibres with different MHC isoforms. A mixture of abundant proteins with distinct molecular weights close to those of MHC was detected during the SDS-PAGE of the rat diaphragm, reflecting skeletal muscle fibres of types I( $\beta$ ), IIx, IIb and IIa (Talmadge and Roy, 1993). MHC  $\alpha + \beta$  isoforms of the LV samples co-migrated with MHC  $\beta$  isoforms in skeletal muscle fibres (van der Velden *et al.*, 1999) (Figure 4B). Although we could not extend the mechanical measurements

on isolated muscle fibres to biochemical MHC isoform subtyping, the rate constant of force redevelopment at pCa 4.75 ( $k_{\text{tr,max}}$ ) parameters allowed the differentiation between muscle fibres with slow ( $k_{\text{tr,max}} < 2 \text{ s}^{-1}$ ) and fast intrinsic kinetics ( $k_{\text{tr,max}} > 2 \text{ s}^{-1}$ ), most probably reflecting the isoform compositions of type I vs. type II fibres (Figure 4C). When diaphragm myofibres with distinct kinetic characteristics were analysed separately (on the basis of their  $k_{\text{tr,max}}$  parameters as indicated above), OM-dependent  $\text{Ca}^{2+}$  sensitization



**Figure 4**

The  $\text{Ca}^{2+}$ -sensitizing effect of OM in permeabilized rat diaphragm skeletal muscle fibres. (A) Force recordings illustrated  $\text{Ca}^{2+}$ -induced contractions with increasing amplitudes at a submaximal  $[\text{Ca}^{2+}]$  (pCa 6.0), due to the  $\text{Ca}^{2+}$ -sensitizing effect of OM in an individual permeabilized skeletal muscle fibre. The experimental arrangement is similar to that of carried out in isolated cardiomyocytes. (B) MHC isoform compositions of the muscle fibres in the rat diaphragm (D) and the rat cardiomyocytes (LV). (C) Actin–myosin cross-bridge cycle kinetics were assessed through their  $k_{tr,max}$  parameters in diaphragm muscle fibres under OM-free conditions; the values obtained were scattered within a range between slow ( $k_{tr,max} < 2 \text{ s}^{-1}$ ) and relatively fast kinetics ( $k_{tr,max} > 2 \text{ s}^{-1}$ ). (D) OM exhibited a bell-shaped concentration–isometric force relationship in muscle fibres with slow intrinsic kinetics ( $n = 14$  muscle fibres from six different animals). Increasing concentrations of OM was applied in the same muscle fibres. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett’s two-tailed test.

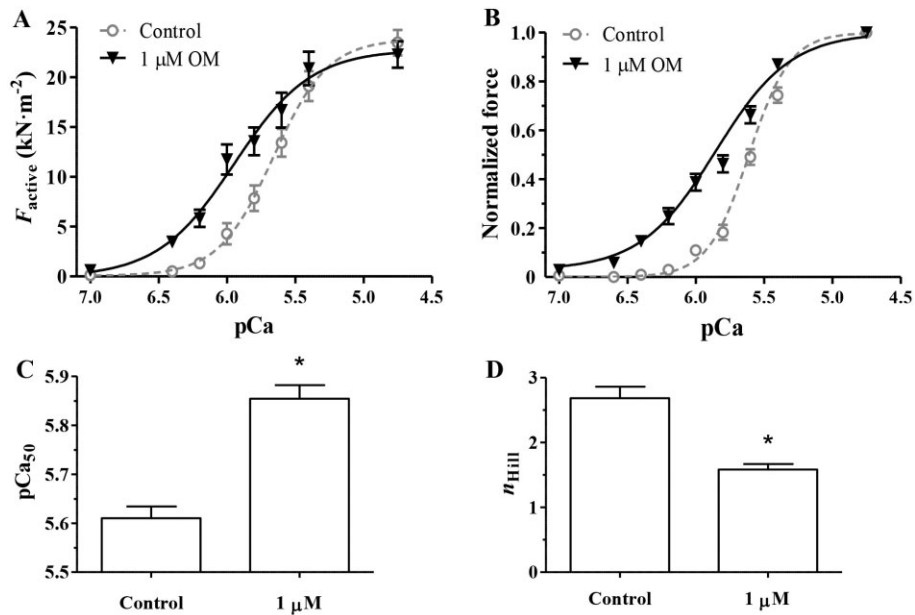
was revealed with an apparent  $\text{EC}_{50}$  of  $0.16 \pm 0.03 \mu\text{M}$  in slow fibres ( $k_{tr,max}$ :  $1.61 \pm 0.22 \text{ s}^{-1}$ ; Figure 4D).

The  $\text{Ca}^{2+}$ -sensitizing effect of OM was demonstrated by comparing pCa–force relationships in diaphragm muscle preparations with low  $k_{tr,max}$  ( $1.50 \pm 0.10 \text{ s}^{-1}$ ) in the absence and presence of OM. Isometric force values were expressed in absolute (Figure 5A) and normalized terms (Figure 5B).  $1 \mu\text{M}$  OM treatment resulted in a pronounced leftward shift of the pCa–normalized force relationship as OM increased the  $\text{Ca}^{2+}$  sensitivity of force production in the isolated rat diaphragm muscle fibres (pCa<sub>50</sub> in OM-free controls:  $5.61 \pm 0.02$  and at  $1 \mu\text{M}$  OM:  $5.85 \pm 0.03$ ;  $P < 0.001$ ; Figure 5C). This OM concentration exerted less effect on the force production at either the highest or the lowest  $[\text{Ca}^{2+}]$  than in isolated cardiomyocytes. Furthermore,  $1 \mu\text{M}$  OM decreased  $n_{Hill}$  of the normalized pCa–force relationships ( $n_{Hill}$  in OM-free medium:  $2.68 \pm 0.18$  and  $1.58 \pm 0.09$  at  $1 \mu\text{M}$  OM;  $P < 0.01$ ; Figure 5D).

### Mechanical and kinetic effects of OM in isolated muscle fibres of rat diaphragm with slow intrinsic kinetics

The effects of OM on the kinetics of  $\text{Ca}^{2+}$ -dependent activations and relaxations were also investigated in isolated diaphragm muscle fibres with slow intrinsic kinetics ( $k_{tr,max}$ :  $1.57 \pm 0.11 \text{ s}^{-1}$ ) at saturating (pCa 4.75) and submaximal (pCa 6.0)

$[\text{Ca}^{2+}]$  similarly to that in cardiomyocytes. Although  $F_{max}$  of the preparations decreased at  $3 \mu\text{M}$  OM and higher at pCa 4.75 ( $F_{active}$  in OM-free medium:  $21.37 \pm 3.16 \text{ kN}\cdot\text{m}^{-2}$  and  $13.25 \pm 1.78 \text{ kN}\cdot\text{m}^{-2}$  at  $3 \mu\text{M}$  OM;  $P < 0.001$  vs. the control; Supporting Information Fig. S1b), OM exhibited a bell-shaped concentration–response relationship due to  $\text{Ca}^{2+}$  sensitization at pCa 6.0, similar to that observed in cardiomyocytes. OM increased  $F_{passive}$  at pCa 4.75 and 6.0 ( $\text{EC}_{50}$ :  $0.79 \pm 0.16 \mu\text{M}$  and  $0.69 \pm 0.11 \mu\text{M}$  at pCa 4.75 and pCa 6.0, respectively; Figure 6A). OM decreased  $k_{tr}$  both at pCa 4.75 and 6.0, although with a higher potency at pCa 4.75 ( $\text{EC}_{50}$ :  $0.13 \pm 0.09 \mu\text{M}$ ) than at pCa 6.0 ( $\text{EC}_{50}$ :  $0.29 \pm 0.04 \mu\text{M}$ ; Figure 6B). The kinetic features of the OM effects were also reflected by increases in  $t_{1/2}$  of activation and  $t_{relax}$  parameters in diaphragm myofibres with low  $k_{tr,max}$ . OM significantly increased the  $t_{1/2}$  of activation at  $1 \mu\text{M}$  and higher concentration both at pCa 4.75 ( $t_{1/2}$  of activation in OM-free medium:  $5.99 \pm 0.98 \text{ s}$  and  $13.98 \pm 1.46 \text{ s}$  at  $1 \mu\text{M}$  OM,  $P < 0.001$  vs. the control) and at pCa 6.0 ( $t_{1/2}$  of activation in OM-free medium:  $6.96 \pm 0.86 \text{ s}$  and  $10.50 \pm 0.93 \text{ s}$  at  $1 \mu\text{M}$  OM,  $P < 0.001$  vs. the control; Figure 6C).  $0.3 \mu\text{M}$  and higher OM slowed the relaxation at pCa 4.75 ( $t_{relax}$  in OM-free medium:  $3.15 \pm 0.68 \text{ s}$  and  $5.49 \pm 0.81$  at  $0.3 \mu\text{M}$  OM s;  $P < 0.05$  vs. the control), whereas at pCa 6.0, similar effects were observed at  $1 \mu\text{M}$  OM and higher concentration ( $t_{relax}$  in OM-free medium:  $0.86 \pm 0.09 \text{ s}$  and  $7.26 \pm 0.83 \text{ s}$  at  $1 \mu\text{M}$  OM;  $P < 0.01$  vs. the control; Figure 6D).



**Figure 5**

The Ca<sup>2+</sup>-sensitizing effect of OM in permeabilized skeletal rat diaphragm muscle fibres with slow kinetics was quantified through pCa–force relationships ([Ca<sup>2+</sup>] between pCa 7.0 and pCa 4.75) in the absence and presence of 1 μM OM. (A, B) Means of force amplitudes are expressed in absolute terms (kN·m<sup>-2</sup>) and normalized terms, fitted to a modified Hill equation. (C) OM-evoked Ca<sup>2+</sup> sensitization and the resultant leftward shift in the isometric pCa–force relations are illustrated by the means of their midpoints (pCa<sub>50</sub>) in the absence and presence of 1 μM OM. (D) OM decreased the co-operativity of Ca<sup>2+</sup>-regulated force production (n<sub>Hill</sub>) in the permeabilized skeletal muscle fibres (n = 9 muscle fibres from four different animals). Test activations in the absence and in the presence of OM were carried out in the same muscle fibres. \*P < 0.05, significantly different from control; paired Student's *t*-test.

### Mechanical and kinetic effect of OM in isolated muscle fibres of rat diaphragm with fast intrinsic kinetics

OM-dependent mechanical and kinetic effects were also assessed in isolated skeletal muscle fibres with fast intrinsic kinetics ( $k_{tr,max}$ :  $4.60 \pm 0.31$  s<sup>-1</sup>). Measurements were carried out at saturating (pCa 4.75) and submaximal (pCa 5.8 at this case) [Ca<sup>2+</sup>].  $F_{max}$  was not affected; however, OM exhibited a significant Ca<sup>2+</sup>-sensitizing effect at 1 μM concentration and higher at pCa 5.8 ( $F_{active}$  in OM-free medium:  $4.59 \pm 0.99$  kN·m<sup>-2</sup> and  $6.97 \pm 1.30$  kN·m<sup>-2</sup> at 1 μM OM;  $P < 0.05$  vs. the control; Supporting Information Fig. S1c). OM treatment did not increase  $F_{passive}$  of fast myofibres either at pCa 4.75 or at pCa 5.8 (Figure 7A). OM decreased the  $k_{tr}$  at 1 μM concentration and higher at pCa 4.75 (EC<sub>50</sub>:  $1.23 \pm 0.28$  μM), but had no effect on  $k_{tr}$  at pCa 5.8 (Figure 7B). The highest OM concentration was associated with faster Ca<sup>2+</sup> contractions at pCa 5.8 ( $t_{1/2}$  of activation in OM-free medium:  $8.33 \pm 0.70$  s and  $4.92 \pm 0.96$  s at 1 μM OM,  $P < 0.01$  vs. the control; Figure 7C), whereas the relaxation of the preparations was not affected following Ca<sup>2+</sup> removal either at pCa 4.75 or at pCa 5.8 (Figure 7D).

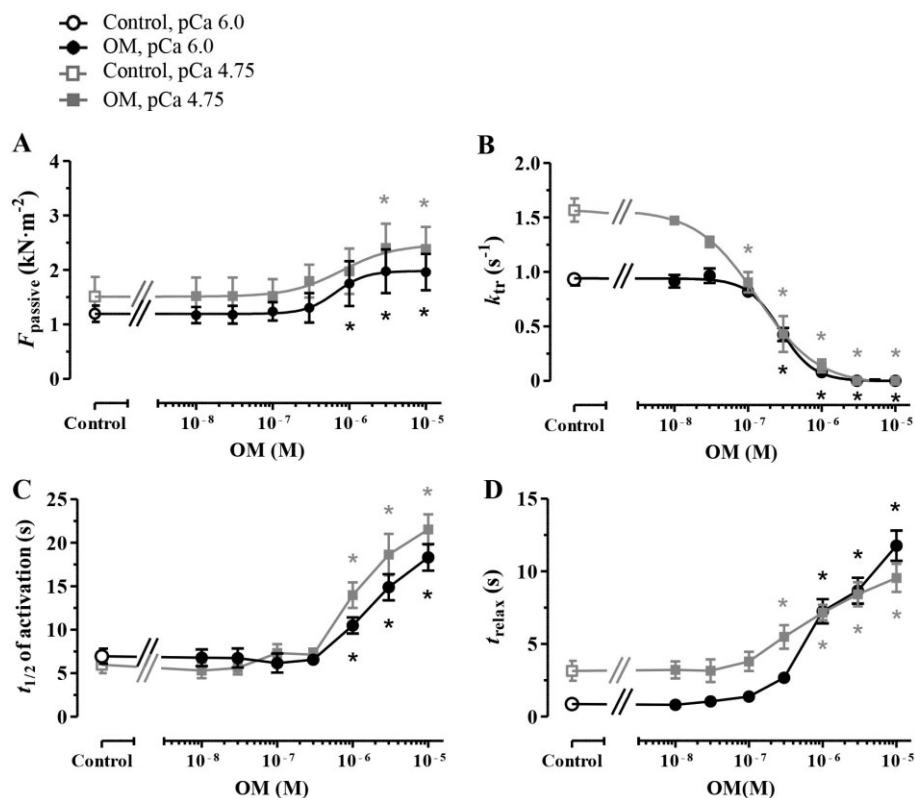
## Discussion

As far as we are aware, this is the first quantitation of the Ca<sup>2+</sup>-sensitizing effect of the novel myosin activator OM in

isolated and skinned cardiomyocytes. OM altered the kinetics of both activations and relaxations in cardiomyocytes during Ca<sup>2+</sup>-induced contractions. The experimental data additionally revealed that OM could also improve the contractile function of the diaphragm by exerting a Ca<sup>2+</sup>-sensitizing effect on force production in skeletal muscle fibres.

Myosin activators are novel sarcomere-targeted agents for the treatment of HF without the deleterious side effects of the traditional inotropes (Teerlink, 2009). OM activates the S1 domain of the cardiac myosin heads by binding to the base of the lever arm where the relay helix and the converter domain converge. This pharmacological intervention increases the ATPase activity of the myosin heads (Malik *et al.*, 2011; Wang *et al.*, 2014). Our present data indicate a similar relationship for the OM-evoked changes in the Ca<sup>2+</sup>-dependence of the contractile force production to that already described for the OM-evoked changes in the Ca<sup>2+</sup> sensitivity of myosin ATPase activity (Malik *et al.*, 2011). It is now increasingly accepted, that OM facilitates P<sub>i</sub> release while leaving ADP dissociation unchanged thereby increasing the available number of cross-bridges during cardiac contractions without affecting the intracellular Ca<sup>2+</sup> homeostasis (Liu *et al.*, 2015). This suggests that myosin activation can be regarded as a downstream Ca<sup>2+</sup>-sensitizing mechanism. In our present study, OM increased the Ca<sup>2+</sup> sensitivity of force production at OM concentrations of 0.1 μM and higher, while OM-dependent changes in ATPase rate and myocardial contractility were also reported at overlapping OM concentrations in preclinical model experiments and clinical trials (Cleland *et al.*, 2011;





**Figure 6**

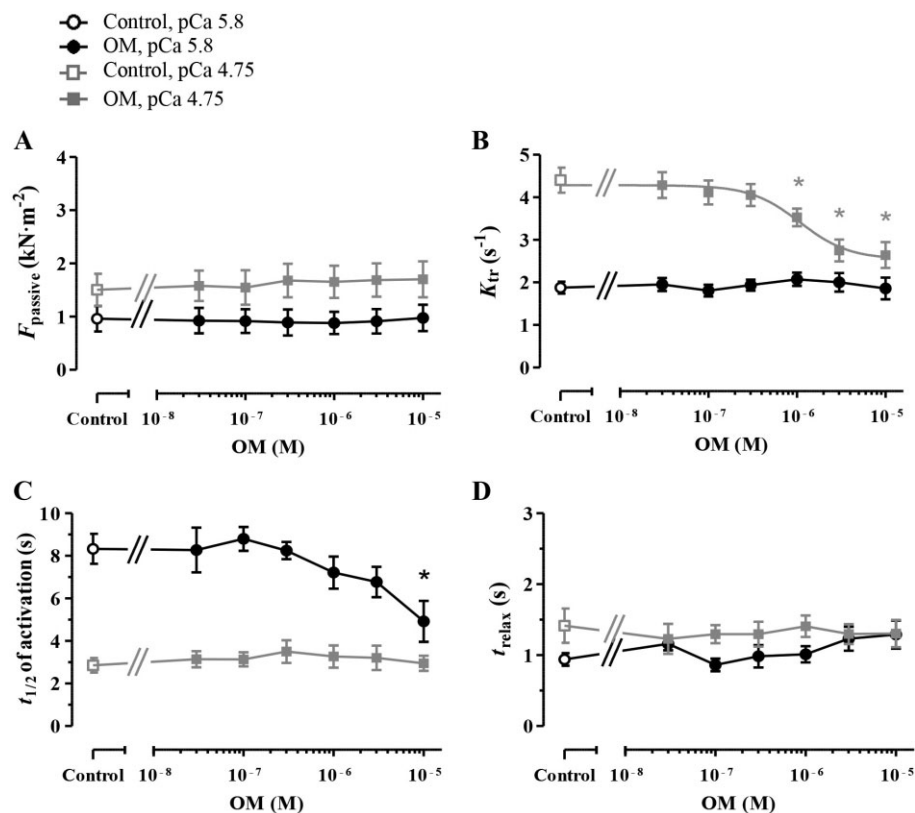
The concentration dependency of OM-induced mechanical and kinetic changes on slow skeletal muscle fibres of the rat diaphragm were illustrated in the function of the applied  $[\text{Ca}^{2+}]$  (pCa 4.75 and pCa 6.0). (A) OM increased  $F_{\text{passive}}$  of the preparations. (B) The OM-evoked decrease of  $k_{\text{tr}}$  was more pronounced at pCa 4.75 than at pCa 6.0. (C, D)  $t_{1/2}$  of activation and  $t_{\text{relax}}$  were prolonged in response to increasing OM concentration in slow fibres,  $n = 6$  muscle fibres at pCa 4.75 and 11–14 muscle fibres at pCa 6.0, from 3–6 different animals respectively). Increasing concentrations of OM was applied in the same muscle fibre preparations. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.

Malik *et al.*, 2011; Teerlink *et al.*, 2011; Wang *et al.*, 2014). These results imply that OM-dependent  $\text{Ca}^{2+}$  sensitization and myosin ATPase activation illustrate different, although closely related facets of the OM-induced sarcomeric changes.

Recruitment of cross-bridges by OM may delay the inactivation of the thin filaments leading to increased number of strongly-attached cross-bridges and prolonged activation of the contractile machinery (Gordon *et al.*, 2000; Poggesi *et al.*, 2005). Our kinetic data on increased  $t_{1/2}$  of activation and decreased  $k_{\text{tr}}$  corroborate the decreased *in vitro* motilities of the myosin filaments (Wang *et al.*, 2014; Liu *et al.*, 2015) and decreased contraction and relaxation velocity of the cardiomyocytes (Malik *et al.*, 2011; Shintani *et al.*, 2014), potentially manifesting in increased systolic ejection time. Additionally, sustained thin filament activation and a reduction in the ATP-initiated dissociation rate of acto-myosin can delay the kinetics of the force decay during diastole as indicated by the changes of  $t_{\text{relax}}$  in this study (Poggesi *et al.*, 2005; Liu *et al.*, 2015). Although the kinetics of force generation *in vitro* were slower, the maximal rate of LV pressure development (i.e.  $dP/dt_{\text{max}}$ ) was not affected by OM (Shen *et al.*, 2010). Perhaps the  $\text{Ca}^{2+}$ -sensitizing effect of OM may partially counterbalance the slowed kinetics of contraction as suggested by

the differences in OM concentration dependencies between maximal and submaximal activations (Figure 3C and 3D). Taken together, OM-evoked  $\text{Ca}^{2+}$  sensitization may result in stronger, slower and prolonged cardiac contractions consistent with the echocardiographic findings (Cleland *et al.*, 2011; Teerlink *et al.*, 2011).

$\text{Ca}^{2+}$  sensitizers affecting the cross-bridge cycling of the myofilaments may alter force production at both high and low  $[\text{Ca}^{2+}]$  (Kass and Solaro, 2006). Accordingly, OM exerted robust  $\text{Ca}^{2+}$ -sensitizing effects over a wide range of  $[\text{Ca}^{2+}]$ , but both the extent and the direction of the evoked changes (force enhancement vs. force mitigation) depended on the applied OM concentration. At 1  $\mu\text{M}$  OM, contractile force development was observed at very low  $[\text{Ca}^{2+}]$  (pCa 7.0–9.0), as suggested by the upward shift in the pCa–force relationships. Similar findings have been reported after *in vitro* administration of EMD-57033 or NEM-S1 (Palmer *et al.*, 1995; Fitzsimons *et al.*, 2001; Papp *et al.*, 2004). EMD-57033, the (+)-enantiomer of EMD-53998 and a known downstream  $\text{Ca}^{2+}$  sensitizer, also acts as an allosteric activator of the myosin motor by binding at the base of the lever arm located close to the proposed binding site of OM (Radke *et al.*, 2014). NEM-S1, the N-ethylmaleimide derivative of myosin S-1 facilitates



## Figure 7

The mechanical and kinetic effects of OM were also tested in permeabilized skeletal muscle fibres of the rat diaphragm with fast intrinsic kinetics at saturating (pCa 4.75) and submaximal (pCa 5.8)  $[\text{Ca}^{2+}]$ . (A)  $F_{\text{passive}}$  of the preparations was not affected by OM treatment. (B) OM decreased  $k_{\text{tr}}$  at pCa 4.75; however, this effect was missing at pCa 5.8. (C)  $t_{1/2}$  of activation was unaltered at pCa 4.75 and decreased at pCa 5.8 in response to the highest applied OM concentration. (D) OM did not influence the relaxation of fast skeletal muscle fibres.  $n = 6 - 6$  isolated myofibres for both  $[\text{Ca}^{2+}]$  from three different animals. Increasing concentrations of OM was applied in the same muscle fibres. \* $P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.

cross-bridge binding to the thin filaments, potentiating thereby the acto-myosin ATPase activity (Swartz and Moss, 2001). Isometric force production by downstream  $\text{Ca}^{2+}$ -sensitizers in the absence of  $\text{Ca}^{2+}$  implies that the thin filaments are not completely switched off without  $\text{Ca}^{2+}$  (Moss *et al.*, 2004). Furthermore, the  $\text{Ca}^{2+}$ -sensitizing effect of OM was absent at pCa 4.75, suggesting that the available number of strongly bound cross-bridges are maximal under this condition (Moss *et al.*, 2004).

An increased  $\text{Ca}^{2+}$  sensitivity of force production has been observed in advanced stages of HF (van der Velden *et al.*, 2003). Moreover,  $\text{Ca}^{2+}$  sensitization at diastolic  $[\text{Ca}^{2+}]$  may be complicated by an already elevated cytosolic  $[\text{Ca}^{2+}]$  during diastole in HF (Webster *et al.*, 1993). We have now demonstrated that OM can exert a robust  $\text{Ca}^{2+}$ -sensitizing effect at diastolic  $[\text{Ca}^{2+}]$  and can additionally increase the  $F_{\text{passive}}$  of the cardiomyocytes. Additionally, OM substantially prolonged the relaxation of the myocyte-sized preparations following the dissociation of  $\text{Ca}^{2+}$  from the contractile apparatus. All of these components may complicate ventricular relaxation during OM treatment especially at high doses. Accordingly, the worsened time constant of isovolumic relaxation ( $\tau$ ) and

rate of the LV pressure decrease ( $dP/dt_{\text{min}}$ ) reflected impaired diastolic performance after i.v. OM administration in rats with volume overload HF (Wilson *et al.*, 2014).

Despite the *in vitro* mechanical and kinetic effects, OM treatment did not impair the diastolic functions in healthy volunteers or in patients with systolic HF at plasma concentrations similar to those applied in this study (approximately 0.25–2.5  $\mu\text{M}$ ; Cleland *et al.*, 2011; Teerlink *et al.*, 2011). It must therefore be acknowledged that extrapolation from *in vitro* data to *in vivo* conditions can be complicated by the distinct interactions of OM with the actin–myosin system in permeabilized preparations and in the structurally intact myocardium (Solaro *et al.*, 1993; Palmer *et al.*, 1995). Through the use of an isolated single-cell experimental arrangement, the contractile machinery was directly exposed to OM, in contrast with the structurally intact fibres, and hence resulting in a potentially greater extent of myosin activation at the applied concentration range than that achieved *in vivo*.

HF is also associated with the decreased contractility of the diaphragm attributable to its decreased  $\text{Ca}^{2+}$  sensitivity (Empinado *et al.*, 2014). Further, mechanical ventilation is accompanied by the impaired contractile performance of the

Table 1

OM concentration-dependent mechanical and kinetic effects in permeabilized cardiomyocytes and diaphragm muscle fibres

	Cardiomyocytes	Diaphragm muscle fibres with low $k_{tr,max}$	Diaphragm muscle fibres with high $k_{tr,max}$
EC <sub>50</sub> for Ca <sup>2+</sup> sensitization of force production	0.08 ± 0.01	0.16 ± 0.03	#
EC <sub>50</sub> for $F_{passive}$ increase	0.26 ± 0.11	0.69 ± 0.11	No effect
EC <sub>50</sub> for $k_{tr}$ decrease	#	0.29 ± 0.04	No effect

EC<sub>50</sub> values (in μM), determined at submaximal [Ca<sup>2+</sup>] (pCa 6.0 or pCa 5.8) for each muscle preparations are shown as means ± SEM. #EC<sub>50</sub> could not be determined.

respiratory muscles (Jaber *et al.*, 2011). Previous studies have revealed that levosimendan and EMD-57033 improved the contractility of the failing diaphragm through their Ca<sup>2+</sup>-sensitizing mechanisms (Ochala *et al.*, 2010; Doorduyn *et al.*, 2012). In the present study, OM exerted its Ca<sup>2+</sup>-sensitizing effect in the skeletal muscle fibres of the rat diaphragm because the slow-skeletal muscle fibres co-express the same MHC isoform (MHC-β) as found in the heart (Bers and Harris, 2011; Schiaffino and Reggiani, 2011). Our present data additionally demonstrated that OM exerted its effect on the Ca<sup>2+</sup> sensitivity of force production and on the kinetic characteristics of the slow diaphragm preparations at a higher concentration range than in isolated cardiomyocytes. Moreover, our data probably suggest that OM may influence the contractility of the muscle fibres with fast skeletal MHCs, although its Ca<sup>2+</sup>-sensitizing potency was even lower in isolated muscle fibres with high  $k_{tr,max}$  than in those developing low  $k_{tr,max}$ , with less effects on  $F_{passive}$  production and the kinetics of activation–relaxation cycles (Table 1). As reported on the ATOMIC-HF trial, the treatment of HF patients with OM may decrease the incidence of dyspnoea (Meijs *et al.*, 2012; Valentova and Von Haehling, 2014). Our present study suggests that this might be due in part to the enhanced contractility of the diaphragm resulting particularly from Ca<sup>2+</sup> sensitization.

One of the limitations of our study relates to the choice of isolated single-cell preparations, in consequence of which the myosin isoform composition of the investigated skeletal muscle preparations had to be addressed through their respective OM-free  $k_{tr,max}$  parameters and not through protein biochemistry. Nevertheless, it has previously been shown that  $k_{tr,max}$  in single-fibre preparations may be appropriate for the differentiation of slow and fast twitching myofibres (Bodnar *et al.*, 2014).

Overall, the findings of the present study have revealed that the mechanism of action of OM-evoked myosin activation is similar to that of Ca<sup>2+</sup>-sensitizing positive inotropes with targets downstream from cTnC in the heart. Moreover, myosin activation may improve the contractility of the diaphragm via a Ca<sup>2+</sup>-sensitizing mechanism, most effectively in muscle fibres with slow intrinsic kinetics.

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## Author contributions

L. N., Á. K., B. B., E.T. P. and G.Á. F. performed the research, A. T., I. É. and Z. P. designed the research study, L. N., Á. K., B. B., G.Á. F., E.T. P. and Z. P. carried out the data and statistical analysis, L. N., A. T., I. É. and Z. P. wrote the paper.

## Conflict of interest

The authors have nothing to disclose.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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**Figure S1** OM-evoked changes in  $F_{\text{active}}$  at saturating  $[\text{Ca}^{2+}]$  and at a submaximal level of activation in cardiomyocytes, in skeletal myofibres with slow intrinsic kinetics and in skeletal myofibres with high intrinsic kinetics. (A,B) In cardiomyocytes and slow skeletal muscle fibres,  $F_{\text{active}}$  decreased linearly at pCa 4.75 with OM concentration, whereas at pCa, 6.0 the OM concentration– $F_{\text{active}}$  relationships were bell-shaped. (C) OM did not affect  $F_{\text{active}}$  at pCa 4.75 while exhibiting a weak  $\text{Ca}^{2+}$ -sensitizing effect at the submaximal  $[\text{Ca}^{2+}]$  in fast skeletal muscle preparations.  $n = 6 - 6$  isolated myofibres from three different animals respectively. Increasing concentrations of OM were applied in the same preparations.  $*P < 0.05$ , significantly different from control; repeated measures ANOVA with Dunnett's two-tailed test.