

# Beneficial effects of SR33805 in failing myocardium

Younss Ait Mou<sup>1,2\*</sup>, Attila Toth<sup>3</sup>, Cécile Cassan<sup>1</sup>, Daniel Czuriga<sup>3</sup>, Pieter P. de Tombe<sup>2</sup>, Zoltan Papp<sup>3</sup>, Alain Lacampagne<sup>1</sup>, and Olivier Cazorla<sup>1</sup>

<sup>1</sup>INSERM U1046, Université Montpellier1, F-34295 Montpellier, France; <sup>2</sup>Department of Cell and Molecular Physiology, Stritch School of Medicine, Loyola University, 2160 South First Avenue, Maywood, Chicago, IL 60153-5500, USA; and <sup>3</sup>Institute of Cardiology, Division of Clinical Physiology, University of Debrecen, Debrecen, Hungary

Received 26 October 2010; revised 25 March 2011; accepted 30 March 2011

Time for primary review: 27 days

## Aims

SR33805, a potent  $\text{Ca}^{2+}$  channel blocker, increases cardiac myofilament  $\text{Ca}^{2+}$  sensitivity in healthy rat cardiomyocytes. Therefore, the aim of the present study was to evaluate the effects of SR33805 on contractile properties in ischaemic failing hearts after myocardial infarction (MI) *in vivo* and *in vitro* at the cellular level.

## Methods and results

The effect of SR33805 (10  $\mu\text{M}$ ) was tested on the excitation–contraction coupling of cardiomyocytes isolated from rat with end-stage heart failure. Cell shortening and  $\text{Ca}^{2+}$  transients were measured in intact cardiomyocytes, while contractile properties were determined in Triton X-100 permeabilized myocytes. Acute treatment with SR33805 restored the MI-altered cell shortening without affecting the  $\text{Ca}^{2+}$  transient amplitude, suggesting an increase of myofilament  $\text{Ca}^{2+}$  sensitivity in MI myocytes. Indeed, a SR33805-induced sensitization of myofilament activation was found to be associated with a slight increase in myosin light chain-2 phosphorylation and a more significant decrease on troponin I (TnI) phosphorylation. Decreased TnI phosphorylation was related to inhibition of protein kinase A activity by SR33805. Finally, administration of a single intra-peritoneal bolus of SR33805 (20 mg/kg) improved end-systolic strain and fractional shortening of MI hearts.

## Conclusion

The present study indicates that treatment with SR33805 improved contractility of ischaemic failing hearts after MI in the rat by selectively modulating the phosphorylation status of sarcomeric regulatory proteins, which then sensitized the myofilaments to  $\text{Ca}^{2+}$ . Our results gave a proof of concept that manipulation of the  $\text{Ca}^{2+}$  sensitivity of sarcomeric regulatory proteins can be used to improve contractility of a failing heart.

## Keywords

Myocytes • Heart failure • Contractile function • Sarcomere • Ventricular function

## 1. Introduction

Cardiotonic agents are indispensable for improving contractile dysfunction in acute heart failure (HF) and in the decompensated end-stage HF.<sup>1</sup> These drugs act on various steps of the cardiac excitation–contraction coupling. Most cardiotonic agents available for clinical practice lead to calcium increase and are referred as  $\text{Ca}^{2+}$  mobilizers.<sup>2</sup> Calcium sensitizers present a class of cardiotonic agents that are capable of producing a positive inotropic effect by acting on the cardiac excitation–contraction coupling process at the level of the myofilaments by modifying  $\text{Ca}^{2+}$  binding to troponin C, thin filament regulatory sites, and/or directly the cross-bridge cycling.<sup>2</sup> Their mechanism of action is not associated with an increase in the intracellular  $\text{Ca}^{2+}$  concentration at therapeutic doses, and thus, does not induce  $\text{Ca}^{2+}$ -related deleterious effects such as arrhythmias or apoptosis.<sup>3</sup>

We have previously shown in intact control rat ventricular myocytes that SR33805, a cardiotonic-like agent, reduced the  $\text{Ca}^{2+}$  transient

amplitude and was able to increase cell shortening due to a pronounced increase in myofilament  $\text{Ca}^{2+}$  sensitivity.<sup>4</sup> Interestingly, the  $\text{Ca}^{2+}$  sensitizing effect of the SR33805 was mostly observed when cells were stretched, suggesting a different mechanism of action than other known  $\text{Ca}^{2+}$  sensitizers. SR33805 ([N-[dimethoxy-3,4-phenetyl]-N-methyl-amino-pro-poxyl]-4-benzenesulfonyl]-2-isopropyl-3-methyl-1-indole) is a fantofarone derivative classified as a substituted indole. This compound binds to and inhibits L-type  $\text{Ca}^{2+}$  channels<sup>5</sup> and was characterized as a potent  $\text{Ca}^{2+}$  inhibitor with a favourably weak negative inotropic effect in electrically stimulated rabbit-isolated vascular and cardiac muscle preparations.<sup>6</sup> Similarly, SR33805 blocked the  $\text{Ca}^{2+}$  influx induced by potassium depolarization in porcine coronary artery and maintained force production by inducing a leftward shift of the  $\text{Ca}^{2+}$ –force relationship.<sup>7</sup> The effect of SR33805 on the cardiovascular system has never been tested in pathological conditions.

The phosphorylation of troponin I (TnI) and that of myosin light chain-2 (MLC-2) are known to regulate the cardiac myofilament

\* Corresponding author. Tel: +1 708 216 6245; fax: +1 708 216 6308, Email: yaitmou@lumc.edu

contractility (for review, see Kobayashi and Solaro<sup>8</sup>) by shifting the tension–pCa curve. Modifications of the phosphorylation level of these proteins have been reported during HF and have been proposed to participate in the contractile dysfunction.<sup>9–11</sup> The second hypothesis verified in the present study is whether or not the incubation with the SR33805 could affect the phosphorylation level of contractile regulatory proteins.

We evaluated the effect of SR33805 on, *in vivo* and *in vitro*, contractile properties of a failing rat heart after myocardial infarction (MI). *In vitro*, various indices of excitation–contraction coupling (cell shortening, calcium transient, and myofilament calcium sensitivity) were compared between isolated sham, MI, and SR33805-treated MI cardiomyocytes. Muscle strips were dissected from the left ventricular (LV) free wall and stretched to measure the phosphorylation level of MLC-2 and Tnl, as well as the protein kinase A (PKA) activity. Finally, echocardiography was performed before and after a single intra-peritoneal (ip) bolus of SR33805 in MI hearts to evaluate the potential effects of the drug *in vivo* on global contractile properties.

## 2. Methods

### 2.1 Animals

Male Wistar rats (5 weeks old) were subjected to coronary artery ligation to produce MI as previously described.<sup>9,10</sup> MI rats were compared with sham-operated animals without ligation. Animals were investigated 18 weeks after MI. All experiments complied with the Guide for the 'Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996), with the approval of the French Ministry of Agriculture and according to the European Union Council Directives for care of laboratory animals.

For a detailed description of methods, see the Methods section available as Supplementary material online.

### 2.2 Echocardiography

Cardiac function was assessed using echocardiography (Vivid 7, General Electric Healthcare) with a phase array 10 MHz probe in rats anaesthetized with isoflurane (Baxter) as previously described.<sup>9</sup> The LV end-diastolic or end-systolic diameter and the posterior wall thickness in diastole (PWTd) and systole (PWTs) were measured in M-mode. Fractional shortening (FS) and end-systolic strain (ESS) were calculated using their respective formulas (see Supplementary material online, Methods). A pulsed wave Doppler spectrum of the aortic outflow was recorded using the suprasternal view to evaluate the velocity-time integral (VTI) of the aortic flow. A pulsed wave Doppler spectrum of mitral inflow (E-wave) was recorded using the apical view as an index of diastolic function. The posterior wall velocity was measured with tissue Doppler.

Echocardiography lasted typically 10–15 min per animal. At the end of the first investigation, rats received an ip bolus of SR33805 (20 mg/kg diluted in water) and allowed to recover from anaesthesia. A second echocardiography was performed under the same conditions 30 min after the injection of SR33805. Control experiments were performed to confirm that parameters measured at the second echocardiography in vehicle-treated MI rats were similar to the first echocardiography.

### 2.3 Contractile properties in intact and permeabilized cardiomyocytes

Ventricular myocytes were isolated by enzymatic digestion as previously described<sup>9</sup> from the remaining subendocardium after removal of the fibrotic scar induced by the infarct. Cells loaded with Ca<sup>2+</sup> dye Indo-1 AM (10 μM,

Invitrogen Inc., France) were electrically stimulated at a frequency of 0.5 Hz. Sarcomere length (SL) and fluorescences emitted at 405 and 480 nm were simultaneously recorded using IonOptix system (Hilton, USA) before and after 10 min incubation with 10 μM SR33805.

Measurement of the Ca<sup>2+</sup> sensitivity of force development was performed in myocytes permeabilized with 0.3% Triton X-100 in relaxing solution isolated by mechanical dissociation from subendocardial strips as described previously.<sup>9,10</sup> Myofilaments were incubated with 10 μM SR33805 for 10 min at room temperature after cell attachment.<sup>4</sup> Active and passive forces were measured either by perfusing the cells with different Ca<sup>2+</sup> containing solutions at a given length (1.9 or 2.3 μm SL) or by stretching the cells from 1.9 to 2.3 μm SL in relaxing solution, respectively. Active tensions at submaximal activations were normalized to maximal isometric tension (generally obtained at pCa 5) at the same SL. The relation between force and pCa was fitted using hill equation (see Supplementary material online, Methods for details) from where the Hill coefficient ( $n_H$ ) and pCa for half-maximal activation (pCa<sub>50</sub>) were extracted.

### 2.4 Protein analysis

The phosphorylation levels of Tnl and MLC-2 were performed as previously described<sup>4</sup> in skinned muscle strips treated with ice-cold trichloroacetate (10%) to maintain contractile protein phosphorylation status. In summary, proteins (20 μg) were separated either on 15 or 10% SDS–PAGE for Tnl and MLC-2, respectively. MLC-2 isoforms were specifically detected with a cardiac MLC-2 antibody (Coger SA, Paris, France). Total Tnl content was determined on the first wells of the membrane with a total cardiac Tnl antibody (Cat. #4T21, Hytest, Turku, Finland), and the PKA phosphorylated form of cardiac Tnl antibody on the other wells (Cat. #4T45, Hytest).

PKA activity was measured as described previously.<sup>12</sup> Endogenous PKA activity in the cytosol fraction was measured at room temperature by mixing the cytosol fraction with a phosphorylation solution containing <sup>32</sup>P-labelled ATP (final specific activity: 2500 c.p.m./pmol), 8-Br-cAMP (100 nM), kemptide (1 mg/mL, from GenScript, Piscataway, NJ, USA), and PKA inhibitor (H-89) or SR33805. Radioactivity was measured by scintillation counting (TriCarb scintillation counter from PerkinElmer Life Science, Boston, MA, USA).

Effect of SR33805 on recombinant bovine PKA (Sigma) activity was also tested. The phosphorylation solution contained <sup>32</sup>P-labelled with ATP, PKA inhibitor (H-89 or SR33805), PKA subunit, and histone IIA (1 mg/mL, from Sigma). PKA activities were expressed as % of maximal activity (activity in the absence of inhibitor) over baseline (activity in the absence of kinase) on the figure (Figure 4C).

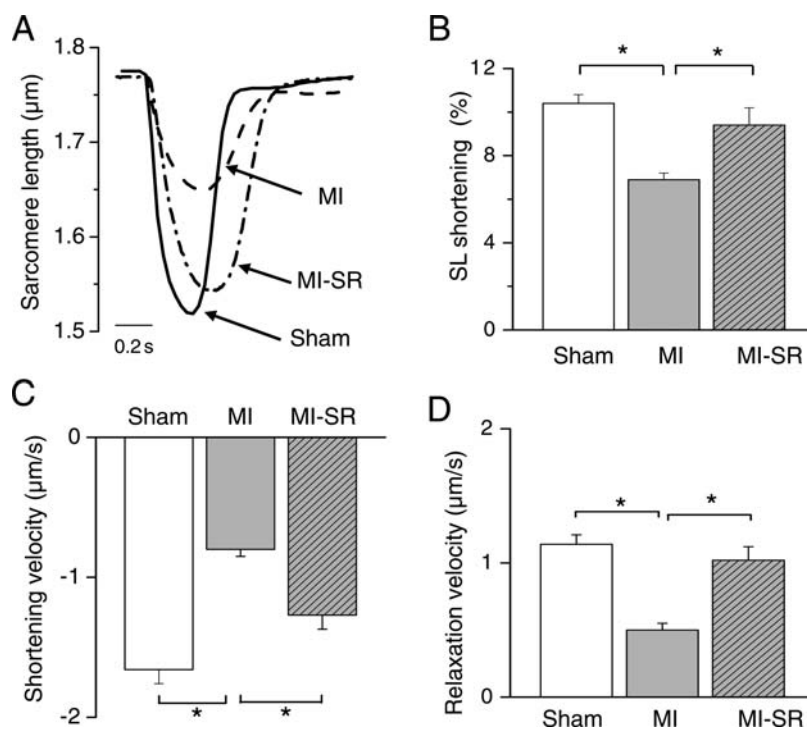
### 2.5 Statistical analysis

One-way ANOVA was applied for comparison between groups. When significant interactions were found, Bonferroni *t*-test was applied with  $P < 0.05$  (Sigmastat 3.5). Data are presented as mean ± SEM.

## 3. Results

### 3.1 Effect of SR33805 on unloaded shortening and Ca<sup>2+</sup> transient of failing myocytes

To test the effect of SR33805 on cell contraction, changes in SL and intracellular Ca<sup>2+</sup> concentration in indo-1-loaded intact cardiomyocytes stimulated at 0.5 Hz were simultaneously recorded. Unloaded contraction decreased significantly with HF associated with a reduction in contraction and relaxation velocities (Figure 1). Incubation of MI cells for 10 min with 10 μM SR33805 restored the amplitude and velocity of SL relaxation (Figure 1). The shortening velocity

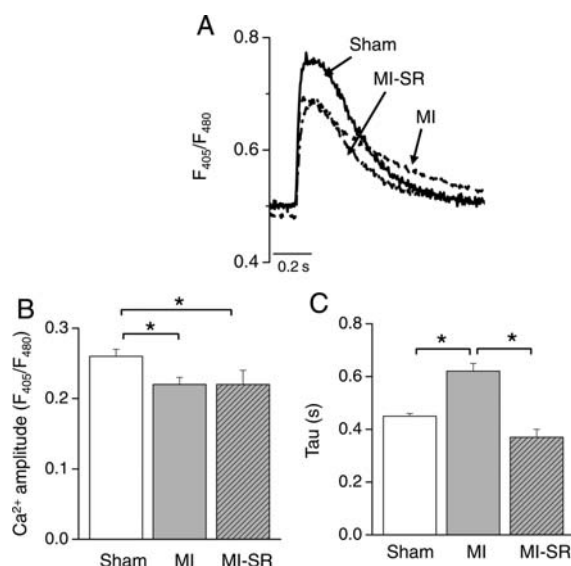


**Figure 1** Effects of SR33805 on SL shortening in isolated MI cardiomyocytes. (A) Representative traces of SL shortening in sham, MI, and MI cells treated with 10  $\mu\text{M}$  SR33805 for 5 min (MI-SR). (B–D) Averaged data of SL shortening amplitude (in % of baseline) (B), SL shortening, and relaxation velocities (C and D, respectively). ( $n = 30\text{--}50$  cells/group, five hearts,  $*P < 0.05$ .)

was partially restored by SR33805. The concentration of the drug used (10  $\mu\text{M}$ ) was determined from our previous study in control rat as the most effective dose on myofilament  $\text{Ca}^{2+}$  sensitivity.<sup>4</sup> The contractile alterations observed in MI cells were associated with a significant decrease in the amplitude of  $\text{Ca}^{2+}$  transient, which was not affected by SR33805 treatment (Figure 2B). The  $\text{Ca}^{2+}$  transient decay was slowed by HF as indicated by the increase in Tau and was normalized by SR33805 (Figure 2C).

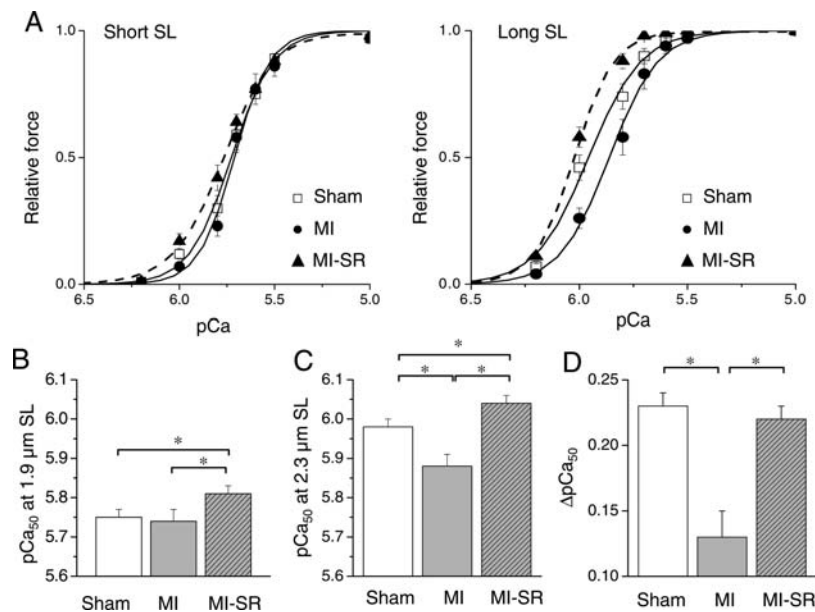
### 3.2 Effect of SR33805 on force development in MI-permeabilized myocytes

Permeabilization of cardiomyocytes allows investigation of the contractile machinery properties and its relation to  $\text{Ca}^{2+}$ , independent of the amount of  $\text{Ca}^{2+}$  released by the sarcoplasmic reticulum. The contractile machinery properties were investigated by measuring the relationship between  $\text{Ca}^{2+}$ -activated tension and  $\text{Ca}^{2+}$  concentration expressed as pCa ( $= -\log[\text{Ca}^{2+}]$ ) in permeabilized cardiomyocytes (Figure 3). Myofilament  $\text{Ca}^{2+}$  sensitivity ( $\text{pCa}_{50}$ ) was measured at short (1.9  $\mu\text{m}$ ) and long (2.3  $\mu\text{m}$ ) SLs (Figure 3A). Myofilament  $\text{Ca}^{2+}$  sensitivity at short SL was not affected by MI and was significantly increased after SR33805 treatment by  $\approx 0.07$  pCa units (Table 1; Figure 3A). Stretching the cells to 2.3  $\mu\text{m}$ , SL induced a leftward shift of tension in both sham and MI groups as indicated by the higher  $\text{pCa}_{50}$  (Figure 3C). However, the shift of the tension–pCa curve after stretch was lower in MI cells, resulting in a lower  $\text{pCa}_{50}$ . This result confirmed previous reports on the same model.<sup>9,10</sup> Myofilament  $\text{Ca}^{2+}$  sensitivity at 2.3  $\mu\text{m}$  SL was improved after SR33805



**Figure 2** Effects of SR33805 on  $\text{Ca}^{2+}$  transient in isolated MI cardiomyocytes. (A) Representative measurement of  $\text{Ca}^{2+}$  transient in sham, MI, and MI cells treated with 10  $\mu\text{M}$  SR33805 for 10 min (MI-SR). (B) Average data of  $\text{Ca}^{2+}$  transient amplitude. (C)  $\text{Ca}^{2+}$  transient decay kinetic as expressed by tau. ( $n = 30\text{--}50$  cells/group, five hearts,  $*P < 0.05$ .)

incubation in MI cells by  $\approx 0.18$  pCa units (Figure 3A and C). The difference between  $\text{pCa}_{50}$  at long and short SL [ $(\Delta\text{pCa}_{50})$ ; used as an index of the length-dependent activation (LDA) of contractile machinery]



**Figure 3** The effect of SR33805 on myofilament Ca<sup>2+</sup> sensitivity in isolated permeabilized MI cardiomyocytes at two SLs: (A) relative tension (normalized to maximal tension)–pCa curves of isolated permeabilized cardiomyocytes were measured at 1.9 (left panel) and at 2.3 μm (right panel) SL in sham cells (square, solid line), MI cells (circle, dashed line), and MI cells treated with SR33805 (MI-SR, triangle, solid line). (B and C) Myofilament Ca<sup>2+</sup> sensitivity at 1.9 μm SL (B) and at 2.3 μm SL (C) was indexed by the measurement of pCa<sub>50</sub> (pCa for half-maximal activation). (D) The stretch-induced Ca<sup>2+</sup> sensitization of activation, ΔpCa<sub>50</sub>, was measured as difference in pCa<sub>50</sub> obtained at 2.3 and 1.9 μm SL. (*n* = 9–15 cells, four hearts, \**P* < 0.05.)

**Table 1** Mechanical properties of sham, MI, and MI incubated with SR33805 (MI-SR) cardiomyocytes

	(n)	Sarcomere length, 1.9 μm				Sarcomere length, 2.3 μm				ΔpCa <sub>50</sub>
		T <sub>pass</sub>	T <sub>max</sub>	pCa <sub>50</sub>	n <sub>H</sub>	T <sub>pass</sub>	T <sub>max</sub>	pCa <sub>50</sub>	n <sub>H</sub>	
Sham	(15)	0.9 ± 0.2	47 ± 4	5.75 ± 0.02	4.1 ± 0.3	11.5 ± 0.7	50 ± 3	5.98 ± 0.02	4.7 ± 0.3	0.23 ± 0.01
MI	(10)	0.9 ± 0.3	40 ± 3	5.74 ± 0.03	4.6 ± 0.5	9.4 ± 1.2	47 ± 3	5.88 ± 0.03 <sup>a</sup>	5.2 ± 0.5	0.13 ± 0.02 <sup>a</sup>
MI-SR	(9)	1.2 ± 0.1	44 ± 3	5.81 ± 0.02 <sup>ab</sup>	3.3 ± 0.1 <sup>b</sup>	12.3 ± 1.5	45 ± 3	6.04 ± 0.02 <sup>ab</sup>	5.5 ± 0.5	0.22 ± 0.01 <sup>b</sup>

Passive (T<sub>pass</sub>) and maximal active (T<sub>max</sub>) tensions in mN/mm<sup>2</sup> were measured at pCa 9 and 4.5, respectively, at 1.9 and 2.3 μm sarcomere length, on cells isolated from sham or MI hearts, with or without 10 min incubation with 10 μM SR33805 (MI-SR). pCa<sub>50</sub> (pCa for half-maximal activation) and n<sub>H</sub> (Hill coefficient) were calculated by fitting the force–pCa relation (see materials and methods). Values are means ± SEM. <sup>a</sup>vs. sham, <sup>b</sup>MI vs. MI-SR; *P* < 0.05.

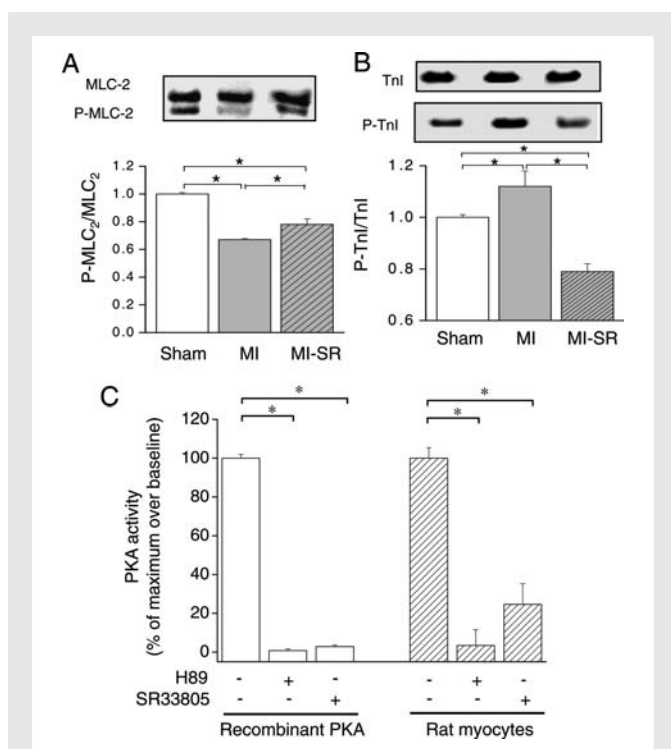
was completely restored following the incubation of MI cells with the SR33805 due to the stronger effect of the drug at long SL (Figure 3D). The other contractile parameters such as passive tension and maximal isometric tension measured at both SL were similar between all groups (Table 1).

### 3.3 Contractile protein phosphorylation

To verify whether the SR33805 effect is associated with a modification in the phosphorylation level of regulatory proteins, the Tnl and MLC-2 phosphorylation levels were measured in stretched permeabilized muscle strips by western blot analysis (Figure 4). HF reduced the phosphorylation level of MLC-2 by ≈33% (Figure 4A), consistent with previous reports from our group.<sup>9,10</sup> SR33805 significantly increased the MLC-2 phosphorylation level in MI tissues. The level of phosphorylation, however, remained 22% lower than in sham cells. For Tnl, a specific antibody that recognizes the phosphorylated sites on Ser<sup>23/24</sup> (the major cAMP-dependent PKA sites) was

used. Tnl phosphorylation was increased in the MI myofilaments (Figure 4B). Incubation with SR33805 significantly reduced the Tnl phosphorylation when compared with MI (≈−30%) and sham (≈−20%) cells (Figure 4B). The reduction of Tnl phosphorylation level observed with the SR33805 treatment suggested either an increase or a decrease of phosphatase and PKA activity, respectively. To this aim, the effect of SR33805 on the PKA activity was measured with a radioactive activity assay (Figure 4C). A recombinant PKA was first used to test a possible direct interaction of the drug with the kinase. The activity of recombinant PKA was decreased by 10 μM SR33805 to a similar extent than that by H89, a standard PKA inhibitor. This result suggested that SR33805 can directly inhibit PKA *in vitro*. This inhibitory effect of SR33805 was also tested on the endogenous PKA activity of the control rat hearts (Figure 4C).

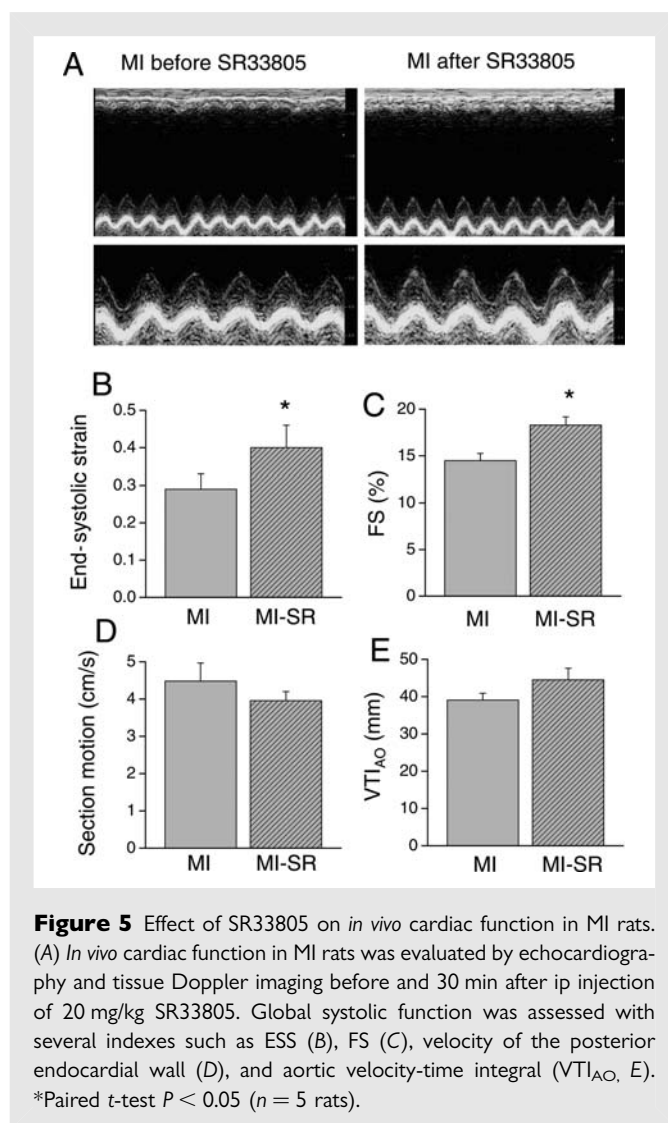
In summary, SR33805 targeted the phosphorylation of Ser<sup>23/24</sup> in Tnl by inhibiting the PKA activity and resulting in an increase in myofilament Ca<sup>2+</sup> sensitivity.



**Figure 4** The effect of SR33805 on sarcomeric protein phosphorylation levels in MI cells: (A) phosphorylated and non-phosphorylated forms of MLC-2 were separated on urea gel and were revealed by western blot analysis with an anti-cardiac MLC-2 antibody. Experiments were performed in stretched muscle strips from sham, MI, and MI treated with SR33805. (B) Western immunoblots with an anti-cardiac TnI antibody (TnI) and anti-PKA-mediated phosphorylated TnI antibody (P-TnI). The phosphorylation level of each protein was expressed relative to the total amount of the protein and was normalized to the level obtained in sham samples ( $n = 5$  hearts). (C) Effect of SR33805 (10  $\mu$ M) on PKA activity was determined by a radioactive assay using either a recombinant PKA or endogenous PKA contained in the control rat hearts. H89 is a PKA inhibitor that was used as a control to block PKA activity. (50 nM,  $n = 3$  experiments,  $*P < 0.05$ ).

### 3.4 *In vivo* cardiac function after SR33805 treatment

Based on the potency of SR33805 to improve contractility at the cellular level in rat hearts after MI, the effect of the drug *in vivo* on global cardiac contractile properties was tested by measuring the cardiac functional parameters by echocardiography and tissue Doppler imaging before and after (30 min) SR33805 treatment (ip injection). A pre-test was performed on rats with 0.2, 2, and 20 mg/kg of SR33805. The highest dose was the only one that had an effect and was selected for completing the *in vivo* test. MI animals had large anterolateral infarcts as indicated by akinetic infarct zone (Figure 5A). Acute treatment with SR33805 increased significantly both ESS and FS by about +38 and +26%, respectively (Figure 5B and C). SR33805 did not affect other contractile parameters such as the kinetics of contraction as determined by the posterior wall motion (Figure 5D) or another index of systolic function, the VTI of the aortic flow (VTI<sub>AO</sub>;  $P = 0.07$ ) (Figure 5E). The E-wave, an index of



**Figure 5** Effect of SR33805 on *in vivo* cardiac function in MI rats. (A) *In vivo* cardiac function in MI rats was evaluated by echocardiography and tissue Doppler imaging before and 30 min after ip injection of 20 mg/kg SR33805. Global systolic function was assessed with several indexes such as ESS (B), FS (C), velocity of the posterior endocardial wall (D), and aortic velocity-time integral (VTI<sub>AO</sub>, E). \*Paired *t*-test  $P < 0.05$  ( $n = 5$  rats).

diastolic function, was not altered by SR33805 between MI ( $1.18 \pm 0.06$  m/s) and MI-SR ( $1.09 \pm 0.04$  m/s).

## 4. Discussion

The present study was designed to test the effect of SR33805 on the cardiac contractile properties of rat with end-stage ischaemic HF characterized by defects of cell shortening, Ca<sup>2+</sup> homeostasis, and myofilament Ca<sup>2+</sup> sensitivity.<sup>9,10</sup> *In vivo*, a single ip bolus of SR33805 in MI rats improved contractile function. This effect could be explained, at least in part, by an effect of the drug at the cardiomyocyte level. Application of 10  $\mu$ M of the SR33805 restored MI cell shortening without affecting the amplitude of Ca<sup>2+</sup> transient. This positive inotropic effect of SR33805 can be explained by the observed increase in myofilament Ca<sup>2+</sup> sensitivity, which could be related to the concomitant slight increase in MLC-2 phosphorylation and a more significant decrease in TnI phosphorylation after PKA inhibition induced by the drug.

It has been shown that increasing contractility after MI worsens cardiac injury and pump dysfunction.<sup>13</sup> However, the increase in contractility was performed by over-expressing of  $\beta$ 2-subunit of the L-type calcium channel, which increased Ca<sup>2+</sup> release. This

therapeutic strategy is the opposite of that typically used clinically for treatment of HF, which involves the use of  $\beta$ -blockers to preserve the reserve of pump function and reduce  $\text{Ca}^{2+}$ -induced arrhythmias. There is, however, a need for cardiotoxic agents for the improvement of contractile dysfunction in acute HF and in the aggravating phases of chronic congestive HF<sup>14</sup> that targets specifically the myofilaments. Recently, it was shown that increasing myofilament calcium sensitivity by over-expressing the slow skeletal TnI isoform delays decompensated hypertrophy following MI.<sup>15</sup> To our knowledge, this is the first study that investigated the effect of the SR33805 in pathological conditions. The results report that an ip bolus of SR33805 can improve *in vivo* LV contractility of rats with HF. *In vivo* LV contractility was estimated by echocardiography investigations. Although these experiments should be confirmed in the future with systemic haemodynamic investigations, they are in line with the results obtained in anaesthetized, open-chest dogs showing that intravenous administration of SR33805 resulted in a large, significant increase in the percentage segment shortening assessed using ultrasonic microcrystals and  $\text{dP/dt}_{\text{max}}$ .<sup>6</sup> This effect cannot be explained by the vasorelaxant and  $\text{Ca}^{2+}$  entry blocker properties of the SR33805 classically described with this drug. Further investigations are required to clearly discriminate the relative contribution of the beneficial effects of this drug on cellular properties and other potential mechanisms. In particular, SR33805 has a strong effect on vascular system which has not been explored in the present study. Part of the positive inotropic effect observed here and in various studies could be related to increased coronary blood flow as described in the dog.<sup>6</sup> Interestingly, at the cellular level, SR33805 did not affect the  $\text{Ca}^{2+}$  transient in failing myocytes, whereas it did in control cells.<sup>4</sup> This effect could be due to a high dependency of SR33805  $\text{Ca}^{2+}$  blockade on membrane potential. Both in vascular<sup>6</sup> and cardiac<sup>16</sup> preparations, SR33805  $\text{Ca}^{2+}$  blockade is reduced in depolarized preparations, which could be the case in failing cardiomyocytes.<sup>17,18</sup> Alternatively, HF-specific alterations in the intracellular metabolic environment can possibly also underlie the difference in the effects of SR33805 on the  $\text{Ca}^{2+}$  transient between failing and control cardiomyocytes.

A potential risk of  $\text{Ca}^{2+}$  sensitizers is the amplification of the diastolic dysfunction induced by an increase in  $\text{Ca}^{2+}$  sensitivity to diastolic levels of  $[\text{Ca}^{2+}]_i$ .<sup>19,20</sup> Previous studies in isolated myocytes and myocardium have demonstrated longer relaxation time, higher resting force,<sup>21</sup> or shorter diastolic cell length<sup>22</sup> after EMD 57033 administration, all of them support myofilament activation. It has been argued that the use of  $\text{Ca}^{2+}$  sensitizers in patients with HF might be deleterious rather than advantageous based on these results. The only class of  $\text{Ca}^{2+}$  sensitizer that reached clinical investigations is levosimendan, which did not alter the relaxation rate<sup>23</sup> and even showed some lusitropic effects in both normal and failing dogs.<sup>24</sup> The goal of the present study was not to compare to or compete with levosimendan but instead to find a drug that could have the same final beneficial effects with reduced negative side effects.

In the present study, there was no sign of relaxation prolongation after SR33805 treatment *in vivo* as assessed by the deceleration time ( $52.6 \pm 6.0$  and  $48.8 \pm 3.8$  ms in MI and MI-SR, respectively). At the cellular level, no signs of increased passive tension in permeabilized myocytes or prolongation of  $\text{Ca}^{2+}$  decline at low stimulation frequency in intact myocytes were observed, but instead, a faster decline as assessed by Tau was obtained, suggesting a favoured  $\text{Ca}^{2+}$  reuptake by SR33805 treatment (Figure 2C). Moreover, SR33805 induced an acceleration of contraction and relaxation

velocities in MI cells (Figure 1C and D) and in control cells.<sup>4</sup> Similar changes on contraction have been reported in myocytes over-expressing the specific cardiac MLCK in which MLC-2 phosphorylation was increased.<sup>25</sup> This suggests that changes of myofilament properties can affect the velocities of cell shortening/relaxation and that the increased phosphorylation level of MLC-2 after SR33805 treatment, although modest, may participate in this effect. In addition, the faster  $\text{Ca}^{2+}$  decay observed in the present study after SR33805 treatment may also participate in the faster relaxation velocity. The mechanism of the accelerated  $\text{Ca}^{2+}$  decay seen after SR33805 treatment is unclear. Calcium transient decay is the resultant of  $\text{Ca}^{2+}$  release (via the ryanodine receptors) and  $\text{Ca}^{2+}$  removal from the cytosol. The latter is performed by SR  $\text{Ca}^{2+}$  reuptake (via SERCA),  $\text{Ca}^{2+}$  extrusion (via sodium-calcium exchange channels), and  $\text{Ca}^{2+}$  binding to the myofilaments. Moreover, the action potential duration (APD) affects the duration of calcium entry through  $\text{I}_{\text{CaL}}$ . In previous experiments, we have shown that SR33805 can reduce the APD by decreasing  $\text{I}_{\text{CaL}}$ . During HF, APD is prolonged, which prolongs  $\text{Ca}^{2+}$  entry via  $\text{I}_{\text{CaL}}$  and alters  $\text{Ca}^{2+}$  signalling duration (for review, see Richard et al.<sup>26</sup>). Although the experiments have not been performed in MI myocytes, we expect APD reduction in MI myocytes with SR33805 treatment, which may initiate earlier  $\text{Ca}^{2+}$  decay without affecting the amplitude of  $\text{Ca}^{2+}$  transient that was already reduced in MI myocytes by  $-15\%$  compared with sham myocytes. More experiments are required to determine the effect of SR33805 on  $\text{Ca}^{2+}$  homeostasis during HF.

An interesting feature of SR33805 is the length dependency of its effects on HF myofilament properties. We observed a modest, but significant, effect at short SL on myofilament  $\text{Ca}^{2+}$  sensitivity ( $+0.07$  pCa unit) and a more pronounced effect at long SL ( $+0.16$  pCa unit), resulting in a normalized LDA as indexed by  $\Delta\text{pCa}_{50}$ . This result had been previously reported in control cells<sup>4</sup> and is not shared with other  $\text{Ca}^{2+}$  sensitizers such as EMD 57033, CGP-48506, and calmidazolium that blunted LDA in permeabilized preparations.<sup>27</sup> The LDA mechanism is a cellular basis of the Frank-Starling law of the heart and is a prerequisite of the normal heart function. The molecular mechanism of LDA and the effect of SR33805 on LDA are unclear. Increasing the phosphorylation level of MLC-2, even to a modest amount, is known to increase myofilament  $\text{Ca}^{2+}$  sensitivity<sup>28</sup> and has been proposed to participate in the LDA regulation.<sup>9,10</sup> In the present study, MLC-2 phosphorylation in stretched preparations was decreased in HF rats compared with sham animals and partially restored after SR33805 treatment (Figure 4A). This is in line with a report in porcine coronary artery study showing that application of SR33805 induces a leftward shift of the  $[\text{Ca}^{2+}]_i$ -force relationship correlated with increased MLC-2 phosphorylation.<sup>7</sup> The signalling pathway that leads to MLC-2 phosphorylation, particularly in a stretched state, is unknown. SR33805 may thus act on a stretch-sensitive kinase or phosphatase and/or a phosphorylation site(s) more accessible in a stretched state. SR33805 modestly, but significantly, increased MLC-2 phosphorylation ( $+10\%$ ). Although minor changes of MLC-2 phosphorylation can significantly change myosin head position, and thus the level of myofilament activation (for review, see Morano<sup>29</sup>), we suspect that other mechanisms are involved that significantly contribute to the large myofilament  $\text{Ca}^{2+}$  sensitization induced by SR33805.

$\text{Ca}^{2+}$  sensitizers can improve contraction by affecting troponins and/or directly facilitating cross-bridge cycling.<sup>14</sup> From the present study, a direct effect of SR33805 on troponins or cross-bridge kinetics

cannot be excluded and has to be determined in future experiments. Tnl phosphorylation on PKA sites was increased in the MI myofilaments (Figure 4B), consistent with a hyperadrenergic state and the decrease of myofilament  $\text{Ca}^{2+}$  sensitivity observed in MI at this stage of HF.<sup>30</sup> During HF, the sympathetic nervous system activity increases in order to maintain an adequate heart output.<sup>31</sup> This process leads to increased  $\beta$ -adrenergic receptor stimulation and PKA-mediated phosphorylation of several excitation–contraction coupling proteins.<sup>32–34</sup> In the advanced stage of HF, the increased sympathetic nervous system leads to a desensitization of  $\beta$ -adrenergic receptors that induces down-regulation of the PKA signalling pathway (for review, see Hamdani et al.<sup>35</sup>). The SR33805 behaved as a  $\beta$ -blocker molecule by blocking PKA activity, which decreased Tnl phosphorylation on PKA sites and could explain part of the sensitization of the myofilaments to calcium.<sup>30</sup> The effect of SR33805 on Tnl is unique in the family of  $\text{Ca}^{2+}$  sensitizers that are known to affect, for some of them, Tnl phosphorylation on PKA sites by the inhibition of phosphodiesterase activity.<sup>36,37</sup> In addition, recent publications showed that  $\text{P}^{21}$ -activated kinase-1 (Pak-1) activation can increase myofilament  $\text{Ca}^{2+}$  sensitivity via stimulation of the phosphatase PP2A that dephosphorylates Tnl.<sup>38,39</sup> Thus, the effect of the SR33805 on phosphatase activity remains to be determined and the possible involvement of Pak-1 should be considered. Nevertheless, the present data suggested a direct inhibition of (recombinant) PKA, which does not support the involvement of these pathways in the SR33805-mediated alterations.

Potential limitations should be acknowledged in this study. In particular, the effect of the SR33805 in human tissue with HF has not been tested here. In human failing heart, several studies have reported an increase in myofilament  $\text{Ca}^{2+}$  sensitivity associated with a decrease in PKA-dependent phosphorylation of Tnl that is due to the heavy medication of the patients in particular with  $\beta$ -blockers.<sup>40–42</sup> Considering that SR33805 affects the level of phosphorylation of several myofilament regulatory proteins, future tests on human tissue with HF should carefully take into account a possible interaction with the pharmacological treatments of the patients.

In summary, our findings show that acute treatment with SR33805 improved cardiac contractility of rat with HF in part by modulating the phosphorylation status of sarcomeric regulatory proteins, which then sensitized the myofilaments to  $\text{Ca}^{2+}$ . Further investigations need to be conducted in order to explore other cellular mechanisms, such as direct interaction of SR33805 with thin or thick filaments, and the relative contribution of the vascular and myocardial systems that may underlay the *in vivo* beneficial effect of the SR33805. Another interesting feature of SR33805 is its lack of deleterious effects on  $\text{Ca}^{2+}$  homeostasis such as increased  $\text{Ca}^{2+}$  release and/or delayed  $\text{Ca}^{2+}$  reuptake. SR33805 might therefore be considered as a therapeutic tool for the management of acute or chronic failing hearts, in particular, those without  $\beta$ -blocker treatment.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

## Acknowledgements

We thank Patrice Bideaux for his technical assistance. SR33805 was a kind gift from Dr P. Gautier (Sanofi-Synthelabo, Montpellier, France). A.L. and O.C. are scientists from the Centre National de Recherche Scientifique.

**Conflict of interest:** none declared.

## Funding

This work was supported by INSERM (Institut National de la Sante et de la Recherche Medicale) and by a research grant from the Franco-Hungarian Partenariat Hubert Curien Balaton program. Y.A.M. and P.d.T. were supported by NIH grant NIH HL62426.

## References

- Endoh M, Hori M. Acute heart failure: inotropic agents and their clinical uses. *Expert Opin Pharmacother* 2006;**7**:2179–2202.
- Endoh M. Mechanism of action of  $\text{Ca}^{2+}$  sensitizers—update 2001. *Cardiovasc Drugs Ther* 2001;**15**:397–403.
- Sorsa T, Pollesello P, Solaro RJ. The contractile apparatus as a target for drugs against heart failure: interaction of levosimendan, a calcium sensitizer, with cardiac troponin c. *Mol Cell Biochem* 2004;**266**:87–107.
- Cazorla O, Lacampagne A, Fauconnier J, Vassort G. SR33805, a  $\text{Ca}^{2+}$  antagonist with length-dependent  $\text{Ca}^{2+}$  -sensitizing properties in cardiac myocytes. *Br J Pharmacol* 2003;**139**:99–108.
- Chatelain P, Dewinkleer P, Beaufort P, Meysmans L, Clinet M. Characterization of the binding of [3H]SR 33805 to the slow  $\text{Ca}^{2+}$  channel in rat heart sarcolemmal membrane. *Eur J Pharmacol* 1994;**267**:151–160.
- Chatelain P, Clinet M, Polster P, Christophe B, Manning AS. *In vitro* characterization of a novel  $\text{Ca}^{2+}$  entry blocker: SR 33805. *Eur J Pharmacol* 1993;**246**:181–193.
- Ieiri S, Hirano K, Nishimura J, Suita S, Kanaide H. Alteration of the  $[\text{Ca}^{2+}]_i$ -force relationship during the vasorelaxation induced by a  $\text{Ca}^{2+}$  channel blocker SR33805 in the porcine coronary artery. *Br J Pharmacol* 2000;**131**:1597–1606.
- Kobayashi T, Solaro RJ. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol* 2005;**67**:39–67.
- Ait Mou Y, Reboul C, Andre L, Lacampagne A, Cazorla O. Late exercise training improves non-uniformity of transmural myocardial function in rats with ischaemic heart failure. *Cardiovasc Res* 2009;**81**:555–564.
- Cazorla O, Szilagyai S, Le Guennec JY, Vassort G, Lacampagne A. Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *FASEB J* 2005;**19**:88–90.
- Kobayashi T, Jin L, de Tombe PP. Cardiac thin filament regulation. *Pflugers Arch* 2008;**457**:37–46.
- Edes IF, Toth A, Csanyi G, Lomnicka M, Chlopicki S, Edes I et al. Late-stage alterations in myofibrillar contractile function in a transgenic mouse model of dilated cardiomyopathy (Tgalphaq<sup>44</sup>). *J Mol Cell Cardiol* 2008;**45**:363–372.
- Zhang H, Chen X, Gao E, MacDonnell SM, Wang W, Kolpakov M et al. Increasing cardiac contractility after myocardial infarction exacerbates cardiac injury and pump dysfunction. *Circ Res* 2010;**107**:800–809.
- Endoh M. Cardiac  $\text{Ca}^{2+}$  signaling and  $\text{Ca}^{2+}$  sensitizers. *Circ J* 2008;**72**:1915–1925.
- Shioura KM, Farjah M, Geenen DL, Solaro RJ, Goldspink PH. Myofilament calcium sensitization delays decompensated hypertrophy differently between the sexes following myocardial infarction. *Am J Physiol Regul Integr Comp Physiol* 2011;**300**:R361–R368.
- Romey G, Lazdunski M. Effects of a new class of calcium antagonists, SR33557 (fantomafarone) and SR33805, on neuronal voltage-activated  $\text{Ca}^{++}$  channels. *J Pharmacol Exp Ther* 1994;**271**:1348–1352.
- Bokenes J, Aronsen JM, Birkeland JA, Henriksen UL, Louch WE, Sjaastad I et al. Slow contractions characterize failing rat hearts. *Basic Res Cardiol* 2008;**103**:328–344.
- Kaprielian R, Wickenden AD, Kassiri Z, Parker TG, Liu PP, Backx PH. Relationship between  $\text{K}^{+}$  channel down-regulation and  $[\text{Ca}^{2+}]_i$  in rat ventricular myocytes following myocardial infarction. *J Physiol* 1999;**517**(Pt 1):229–245.
- Lee JA, Allen DG. EMD 53998 sensitizes the contractile proteins to calcium in intact ferret ventricular muscle. *Circ Res* 1991;**69**:927–936.
- White J, Lee JA, Shah N, Orchard CH. Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic  $\text{Ca}^{2+}$  in isolated ferret cardiac muscle. *Circ Res* 1993;**73**:61–70.
- Hajjar RJ, Schmidt U, Helm P, Gwathmey JK.  $\text{Ca}^{++}$  sensitizers impair cardiac relaxation in failing human myocardium. *J Pharmacol Exp Ther* 1997;**280**:247–254.
- Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N et al. Stereoselective actions of thiazidinones on canine cardiac myocytes and myofilaments. *Circ Res* 1993;**73**:981–990.
- Lehtonen LA. Levosimendan: a parenteral calcium-sensitizing drug with additional vasodilatory properties. *Expert Opin Investig Drugs* 2001;**10**:955–970.
- Masutani S, Cheng HJ, Hyttila-Hopponen M, Levijoki J, Heikkila A, Vuorela A et al. Orally available levosimendan dose-related positive inotropic and lusitropic effect in conscious chronically instrumented normal and heart failure dogs. *J Pharmacol Exp Ther* 2008;**325**:236–247.
- Chan JY, Takeda M, Briggs LE, Graham ML, Lu JT, Horikoshi N et al. Identification of cardiac-specific myosin light chain kinase. *Circ Res* 2008;**102**:571–580.

26. Richard S, Perrier E, Fauconnier J, Perrier R, Pereira L, Gomez AM et al. 'Ca(2+)-induced Ca(2+) entry' or how the L-type Ca(2+) channel remodels its own signaling pathway in cardiac cells. *Prog Biophys Mol Biol* 2006;**90**:118–135.
27. Arteaga GM, Palmiter KA, Leiden JM, Solaro RJ. Attenuation of length dependence of calcium activation in myofilaments of transgenic mouse hearts expressing slow skeletal troponin I. *J Physiol* 2000;**526**(Pt 3):541–549.
28. Pi Y, Zhang D, Kemnitz KR, Wang H, Walker JW. Protein kinase C and A sites on troponin I regulate myofilament Ca<sup>2+</sup> sensitivity and ATPase activity in the mouse myocardium. *J Physiol* 2003;**552**:845–857.
29. Morano I. Tuning the human heart molecular motors by myosin light chains. *J Mol Med* 1999;**77**:544–555.
30. Chandra M, Dong WJ, Pan BS, Cheung HC, Solaro RJ. Effects of protein kinase A phosphorylation on signaling between cardiac troponin I and the N-terminal domain of cardiac troponin C. *Biochemistry* 1997;**36**:13305–13311.
31. Landmesser U, Wollert KC, Drexler H. Potential novel pharmacological therapies for myocardial remodelling. *Cardiovasc Res* 2009;**81**:519–527.
32. Bers DM. Calcium and cardiac rhythms: physiological and pathophysiological. *Circ Res* 2002;**90**:14–17.
33. Kruger M, Linke WA. Protein kinase-A phosphorylates titin in human heart muscle and reduces myofibrillar passive tension. *J Muscle Res Cell Motil* 2006;**27**:435–444.
34. Wolff MR, Buck SH, Stoker SW, Greaser ML, Mentzer RM. Myofibrillar calcium sensitivity of isometric tension is increased in human dilated cardiomyopathies: role of altered beta-adrenergically mediated protein phosphorylation. *J Clin Invest* 1996;**98**:167–176.
35. Hamdani N, Kooij V, van Dijk S, Merkus D, Paulus WJ, Remedios CD et al. Sarcomeric dysfunction in heart failure. *Cardiovasc Res* 2008;**77**:649–658.
36. Brixius K, Reicke S, Reuter H, Schwinger RH. Effects of the Ca<sup>2+</sup> sensitizers EMD 57033 and CGP 48506 on myocardial contractility and Ca<sup>2+</sup> transients in human ventricular and atrial myocardium. *Z Kardiol* 2002;**91**:312–318.
37. Kristof E, Szigeti G, Papp Z, Bodi A, Ball NA, Walsh RA et al. The effects of levosimendan on the left ventricular function and protein phosphorylation in post-ischemic guinea pig hearts. *Basic Res Cardiol* 1999;**94**:223–230.
38. Ke Y, Wang L, Pyle WG, de Tombe PP, Solaro RJ. Intracellular localization and functional effects of P21-activated kinase-1 (Pak1) in cardiac myocytes. *Circ Res* 2004;**94**:194–200.
39. Ke Y, Butt AG, Swart M, Liu YF, McDonald FJ. COMMD1 downregulates the epithelial sodium channel through Nedd4–2. *Am J Physiol Renal Physiol* 2010;**298**:F1445–F1456.
40. Kaumann A, Bartel S, Molenaar P, Sanders L, Burrell K, Vetter D et al. Activation of beta2-adrenergic receptors hastens relaxation and mediates phosphorylation of phospholamban, troponin I, and C-protein in ventricular myocardium from patients with terminal heart failure. *Circulation* 1999;**99**:65–72.
41. Marston SB, de Tombe PP. Troponin phosphorylation and myofilament Ca<sup>2+</sup>-sensitivity in heart failure: increased or decreased?. *J Mol Cell Cardiol* 2008;**45**:603–607.
42. Hamdani N, Paulus WJ, van Heerebeek L, Borbely A, Boontje NM, Zuidwijk MJ et al. Distinct myocardial effects of beta-blocker therapy in heart failure with normal and reduced left ventricular ejection fraction. *Eur Heart J* 2009;**30**:1863–1872.