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Beneficial effects of SR33805 in failing myocardium

Younss Ait Mou1,2*, Attila Toth3, Cécile Cassan1, Daniel Czuriga3, Pieter P. de Tombe2, Zoltan Papp3, Alain Lacampagne1, and Olivier Cazorla1

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Aims
SR33805, a potent Ca^{2+} channel blocker, increases cardiac myofilament 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 μM) was tested on the excitation–contraction coupling of cardiomyocytes isolated from rat with end-stage heart failure. Cell shortening and 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 Ca^{2+} transient amplitude, suggesting an increase of myofilament 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 Ca^{2+}. Our results gave a proof of concept that manipulation of the 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. 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 Ca^{2+} mobilizers. 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 Ca^{2+} binding to troponin C, thin filament regulatory sites, and/or directly the cross-bridge cycling. Their mechanism of action is not associated with an increase in the intracellular Ca^{2+} concentration at therapeutic doses, and thus, does not induce Ca^{2+}-related deleterious effects such as arrhythmias or apoptosis.

We have previously shown in intact control rat ventricular myocytes that SR33805, a cardiotonic-like agent, reduced the Ca^{2+} transient amplitude and was able to increase cell shortening due to a pronounced increase in myofilament Ca^{2+} sensitivity. Interestingly, the Ca^{2+} sensitizing effect of the SR33805 was mostly observed when cells were stretched, suggesting a different mechanism of action than other known Ca^{2+} sensitizers. SR33805 ([N-(dimetoxy-3,4-phenetyl)-N-methyl-amino-pro-poxy]-4-benzenesulfonyl]-2-isopropyl-3-methyl-1-indole) is a farnesofarone derivative classified as a substituted indole. This compound binds to and inhibits L-type Ca^{2+} channels and was characterized as a potent Ca^{2+} inhibitor with a favourably weak negative inotropic effect in electrically stimulated rabbit-isolated vascular and cardiac muscle preparations. Similarly, SR33805 blocked the Ca^{2+} influx induced by potassium depolarization in porcine coronary artery and maintained force production by inducing a leftward shift of the Ca^{2+}–force relationship. 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...
contractility (for review, see Kobayashi and Solaro\textsuperscript{9}) 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.\textsuperscript{9–11} 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 TnI, 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.\textsuperscript{9,10} MI rats were compared with sham-operated animals without ligature. 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 Publication 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.\textsuperscript{9} 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\textsuperscript{12} from the remaining subendocardial after removal of the fibrotic scar induced by the infarct. Cells loaded with Ca\textsuperscript{2+} dye Indo-1 AM (10 μM, Invitrogen Inc., France) were electrically stimulated at a frequency of 0.5 Hz. Sarcomere length (SL) and fluorences 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\textsuperscript{2+} 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.\textsuperscript{9,10} Myofilaments were incubated with 10 μM SR33805 for 10 min at room temperature after cell attachment.\textsuperscript{9} Active and passive forces were measured either by perfusing the cells with different Ca\textsuperscript{2+} 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 (nH) and pCa for half-maximal activation (pCa\textsubscript{50}) were extracted.

2.4 Protein analysis

The phosphorylation levels of TnI and MLC-2 were performed as previously described\textsuperscript{13} in skinned muscle strips treated with ice-cold trichloroacetic (10%) to maintain contractile protein phosphorylation status. In summary, proteins (20 μg) were separated either on 15 or 10% SDS–PAGE for TnI and MLC-2, respectively. MLC-2 isoforms were specifically detected with a cardiac MLC-2 antibody (Coger SA, Paris, France). Total TnI content was determined on the first wells of the membrane with a total cardiac TnI antibody (Cat. #4T21, Hytest, Turku, Finland), and the PKA phosphorylated form of cardiac TnI antibody on the other wells (Cat. #4T45, Hytest). PKA activity was measured as described previously.\textsuperscript{12} Endogenous PKA activity in the cytosol fraction was measured at room temperature by mixing the cytosol fraction with a phosphorylation solution containing 32P-labelled ATP (final specific activity: 2500 c.p.m./pmol), B-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 32P-labelled with ATP, PKA inhibitor (H-89 or SR33805), PKA subunit, and histone II A (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\textsuperscript{2+} transient of failing myocytes

To test the effect of SR33805 on cell contraction, changes in SL and intracellular Ca\textsuperscript{2+} 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
was partially restored by SR33085. The concentration of the drug used (10 \( \mu M \)) was determined from our previous study in control rat as the most effective dose on myofilament Ca\(^{2+} \) sensitivity.\(^4\) The contractile alterations observed in MI cells were associated with a significant decrease in the amplitude of Ca\(^{2+} \) transient, which was not affected by SR33805 treatment (Figure 2B). The 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 Ca\(^{2+} \), independent of the amount of Ca\(^{2+} \) released by the sarcoplasmic reticulum. The contractile machinery properties were investigated by measuring the relationship between Ca\(^{2+} \)-activated tension and Ca\(^{2+} \) concentration expressed as pCa (\( = -\log[Ca^{2+}] \)) in permeabilized cardiomyocytes (Figure 3). Myofilament Ca\(^{2+} \) sensitivity (pCa\(_{50}\)) was measured at short (1.9 \( \mu m \)) and long (2.3 \( \mu m \)) SLs (Figure 3A). Myofilament 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 \( \mu m \), SL induced a leftward shift of tension in both sham and MI groups as indicated by the higher pCa\(_{50}\) (Figure 3C). However, the shift of the tension–pCa curve after stretch was lower in MI cells, resulting in a lower pCa\(_{50}\). This result confirmed previous reports on the same model.\(^5,10\) Myofilament Ca\(^{2+} \) sensitivity at 2.3 \( \mu m \) SL was improved after SR33805 incubation in MI cells by \( \approx 0.18 \) pCa units (Figure 3A and C). The difference between pCa\(_{50}\) at long and short SL ([\( \Delta \)pCa\(_{50}\)] used as an index of the length-dependent activation (LDA) of contractile machinery)
consistent with previous reports from our group. The other contractile parameters such as passive tension and maximal activation in the phosphorylation level of regulatory proteins, the TnI by inhibiting the PKA activity and resulting in an increase in phosphorylation when compared with MI (≈20%) cells (Figure 4B). The reduction of TnI 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 Ser13/24 in TnI by inhibiting the PKA activity and resulting in an increase in myocardial Ca2+ sensitivity.

Figure 3 The effect of SR33805 on myofilament Ca2+ sensitivity in isolated permeabilized MI cardiomyocytes at two SLs: (A) relative tension (normalized to maximal tension)—pCa curves of isolated myocytes 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 Ca2+ sensitivity at 1.9 μm SL (B) and at 2.3 μm SL (C) was indexed by the measurement of pCa50 (pCa for half-maximal activation). (D) The stretch-induced Ca2+ sensitization of activation, ΔpCa50, was measured as difference in pCa50 obtained at 2.3 and 1.9 μm SL. (n = 9–15 cells, four hearts, *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).

<table>
<thead>
<tr>
<th>Sarcomere length, 1.9 μm</th>
<th>Sarcomere length, 2.3 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n) Tpass Tmax pCa50 nH</td>
<td>Tpass Tmax pCa50 nH ΔpCa50</td>
</tr>
<tr>
<td>Sham (15) 0.9 ± 0.2 47 ± 4 5.75 ± 0.02 4.1 ± 0.3</td>
<td>11.5 ± 0.7 50 ± 3 5.98 ± 0.02 4.7 ± 0.3 0.23 ± 0.01</td>
</tr>
<tr>
<td>MI (10) 0.9 ± 0.3 40 ± 3 5.74 ± 0.03 4.6 ± 0.5</td>
<td>9.4 ± 1.2 47 ± 3 5.88 ± 0.03* 5.2 ± 0.5 0.13 ± 0.02*</td>
</tr>
<tr>
<td>MI-SR (9) 1.2 ± 0.1 44 ± 3 5.81 ± 0.02ab 3.3 ± 0.1ab</td>
<td>12.3 ± 1.5 45 ± 3 6.04 ± 0.02ab 5.5 ± 0.5 0.22 ± 0.01ab</td>
</tr>
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Passive (Tpass) and maximal active (Tmax) tensions in mN/mm² 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); pCa50 (pCa for half-maximal activation) and nH (Hill coefficient) were calculated by fitting the force–pCa relation (see materials and methods). Values are means ± SEM. *vs. sham, **MI vs. MI-SR; P < 0.05.

3.3 Contractile protein phosphorylation

To verify whether the SR33805 effect is associated with a modification in the phosphorylation level of regulatory proteins, the TnI 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.8,10 SR33805 significantly increased the MLC-2 phosphorylation level in MI tissues. The level of phosphorylation, however, remained 22% lower than in sham cells. For TnI, a specific antibody that recognizes the phosphorylated sites on Ser13/24 (the major cAMP-dependent PKA sites) was used. TnI phosphorylation was increased in the MI myofilaments (Figure 4B). Incubation with SR33805 significantly reduced the TnI phosphorylation when compared with MI (≈30%) and sham (≈20%) cells (Figure 4B). 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 Ser13/24 in TnI by inhibiting the PKA activity and resulting in an increase in myocardial Ca2+ sensitivity.
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\textsubscript{AO}; P = 0.07) (Figure 5E). The E-wave, an index of diastolic function, was not altered by SR33805 between MI (1.18 ± 0.06 m/s) and MI-SR (1.09 ± 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\textsuperscript{2+} homoeostasis, and myofilament Ca\textsuperscript{2+} sensitivity. 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 μM of the SR33805 restored MI cell shortening without affecting the amplitude of Ca\textsuperscript{2+} transient. This positive inotropic effect of SR33805 can be explained by the observed increase in myofilament Ca\textsuperscript{2+} 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. However, the increase in contractility was performed by over-expressing of β2-subunit of the L-type calcium channel, which increased Ca\textsuperscript{2+} release. This
therapeutic strategy is the opposite of that typically used clinically for treatment of HF, which involves the use of β-blockers to preserve the reserve of pump function and reduce 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\(^{14}\) that targets specifically the myofilaments. Recently, it was shown that increasing myofilament calcium sensitivity by over-expressing the slow skeletal Tn\(i\) isoform delays decompensated hypertrophy following MI.\(^{15}\) 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 \(\Delta P/dt_{max}\).\(^{6}\) This effect cannot be explained by the vasorelaxant and 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.\(^{6}\) Interestingly, at the cellular level, SR33805 did not affect the Ca\(^{2+}\)-transient in failing myocytes, whereas it did in control cells.\(^{4}\) This effect could be due to a high dependency of SR33805 Ca\(^{2+}\) blockade on membrane potential. Both in vascular\(^{6}\) and cardiac\(^{16}\) preparations, SR33805 Ca\(^{2+}\) blockade is reduced in depolarized preparations, which could be the case in failing cardiomyocytes.\(^{17,18}\) Alternatively, HF-specific alterations in the intracellular metabolic environment can possibly also underlie the difference in the effects of SR33805 on the Ca\(^{2+}\)-transient between failing and control cardiomyocytes.

A potential risk of Ca\(^{2+}\)-sensitizers is the amplification of the diastolic dysfunction induced by an increase in Ca\(^{2+}\)-sensitivity to diastolic levels of Ca\(^{2+}\).\(^{19,20}\) Previous studies in isolated myocytes and myocardium have demonstrated longer relaxation time, higher resting force,\(^{23}\) or shorter diastolic cell length\(^{22}\) after EMD 57033 administration, all of them support myofilament activation. It has been argued that the use of Ca\(^{2+}\)-sensitizers in patients with HF might be deleterious rather than advantageous based on these results. The only class of Ca\(^{2+}\)-sensitizer that reached clinical investigations is levosimendan, which did not alter the relaxation rate\(^{23}\) and even showed some lusitropic effects in both normal and failing dogs.\(^{24}\) The goal of the present study was not to compare 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 ± 6.0 and 48.8 ± 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 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 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.\(^{4}\) Similar changes on contraction have been reported in myocytes over-expressing the specific cardiac MLCK in which MLC-2 phosphorylation was increased.\(^{25}\) 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 Ca\(^{2+}\) decay observed in the present study after SR33805 treatment may also participate in the faster relaxation velocity. The mechanism of the accelerated Ca\(^{2+}\) decay seen after SR33805 treatment is unclear. Calcium transient decay is the resultant of Ca\(^{2+}\) release (via the ryanodine receptors) and Ca\(^{2+}\) removal from the cytosol. The latter is performed by SR Ca\(^{2+}\)-uptake (via SERCA), Ca\(^{2+}\) extrusion (via sodium–calcium exchange channels), and Ca\(^{2+}\) binding to the myofilaments. Moreover, the action potential duration (APD) affects the duration of calcium entry through ICa\(_{A}\). In previous experiments, we have shown that SR33805 can reduce the APD by decreasing ICa\(_{A}\). During HF, APD is prolonged, which prolongs Ca\(^{2+}\) entry via ICa\(_{A}\) and alters Ca\(^{2+}\) signalling duration (for review, see Richard et al.\(^{26}\)). Although the experiments have not been performed in MI myocytes, we expect APD reduction in MI myocytes with SR33805 treatment, which may initiate earlier Ca\(^{2+}\) decay without affecting the amplitude of 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 Ca\(^{2+}\)-homoeostasis 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 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 \(\Delta P_{Ca_{50}}\). This result had been previously reported in control cells\(^{4}\) and is not shared with other Ca\(^{2+}\)-sensitizers such as EMD 57033, CGP-48506, and calmidazolium that blunted LDA in permeabilized preparations.\(^{27}\) 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 Ca\(^{2+}\)-sensitivity\(^{28}\) and has been proposed to participate in the LDA regulation.\(^{7,16}\) In the present study, ML-C2 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 [Ca\(^{2+}\)]-force relationship correlated with increased ML-C2 phosphorylation.\(^{7}\) The signalling pathway that leads to ML-C2 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 ML-C2 phosphorylation (+10%). Although minor changes of ML-C2 phosphorylation can significantly change myosin head position, and thus the level of myofilament activation (for review, see Morano\(^{30}\)), we suspect that other mechanisms are involved that significantly contribute to the large myofilament Ca\(^{2+}\)-sensitization induced by SR33805.

Ca\(^{2+}\)-sensitizers can improve contraction by affecting troponins and/or directly facilitating cross-bridge cycling.\(^{14}\) From the present study, a direct effect of SR33805 on troponins or cross-bridge kinetics
can not be excluded and has to be determined in future experiments. TnI phosphorylation on PKA sites was increased in the MI myofilaments (Figure 4B), consistent with a hyperadrenergic state and the decrease of myofilament Ca$^{2+}$ sensitivity observed in MI at this stage of HF. During HF, the sympathetic nervous system activity increases in order to maintain an adequate heart output. This process leads to increased β-adrenergic receptor stimulation and PKA-mediated phosphorylation of several excitation–contraction coupling proteins. In the advanced stage of HF, the heightened sympathetic nervous system leads to a desensitization of β-adrenergic receptors that induces down-regulation of the PKA signalling pathway (for review, see Hamdani et al.). The SR33805 behaved as a β-blocker molecule by blocking PKA activity, which decreased TnI phosphorylation on PKA sites and could explain part of the sensitization of the myofilaments to calcium. The effect of SR33805 on TnI is unique in the family of Ca$^{2+}$ sensitizers that are known to affect, for some of them, TnI phosphorylation on PKA sites by the inhibition of phosphorydiesterase activity. In addition, recent publications showed that p21-activated kinase-1 (Pak-1) activation can increase myofilament Ca$^{2+}$ sensitivity via stimulation of the phosphatase PP2A that dephosphorylates Tnl. 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 Ca$^{2+}$ sensitivity associated with a decrease in PKA-dependent phosphorylation of TnI that is due to the heavy medication of the patients in particular with β-blockers. 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 sarcromeric regulatory proteins, which then sensitized the myofilaments to 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 Ca$^{2+}$- homeostasis such as increased Ca$^{2+}$ release and/or delayed 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 β-blocker treatment.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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