

## K201 improves aspects of the contractile performance of human failing myocardium via reduction in $\text{Ca}^{2+}$ leak from the sarcoplasmic reticulum

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**Abstract** In heart failure, intracellular  $\text{Ca}^{2+}$  leak from cardiac ryanodine receptors (RyR2s) leads to a loss of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) potentially contributing to decreased function. Experimental data suggest that the 1,4-benzothiazepine K201 (JTV-519) may stabilise RyR2s and thereby reduce detrimental intracellular  $\text{Ca}^{2+}$  leak. Whether K201 exerts beneficial effects in human failing myocardium is unknown. Therefore, we have studied the effects of K201 on muscle preparations from failing human hearts. K201 (0.3  $\mu\text{M}$ ; extracellular  $[\text{Ca}^{2+}]_e$  1.25 mM) showed no effects on contractile function and micromolar concentrations resulted in negative inotropic effects (K201 1  $\mu\text{M}$ ; developed tension  $-9.8 \pm 2.5\%$  compared to control group;  $P < 0.05$ ). Interestingly, K201

(0.3  $\mu\text{M}$ ) increased the post-rest potentiation (PRP) of failing myocardium after 120 s, indicating an increased SR  $\text{Ca}^{2+}$  load. At high  $[\text{Ca}^{2+}]_e$  concentrations (5 mmol/L), K201 increased PRP already at shorter rest intervals (30 s). Strikingly, treatment with K201 (0.3  $\mu\text{M}$ ) prevented diastolic dysfunction (diastolic tension at 5 mmol/L  $[\text{Ca}^{2+}]_e$  normalised to 1 mmol/L  $[\text{Ca}^{2+}]_e$ : control  $1.26 \pm 0.06$ , K201  $1.01 \pm 0.03$ ,  $P < 0.01$ ). In addition at high  $[\text{Ca}^{2+}]_e$ , K201 (0.3  $\mu\text{M}$ ) treatment significantly improved systolic function [developed tension  $+27 \pm 8\%$  (K201 vs. control);  $P < 0.05$ ]. The beneficial effects on diastolic and systolic functions occurred throughout the physiological frequency range of the human heart rate from 1 to 3 Hz. Upon elevated intracellular  $\text{Ca}^{2+}$  concentration, systolic and diastolic contractile functions of terminally failing human myocardium are improved by K201.

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## Introduction

Patients with chronic heart failure (HF) exhibit depressed contractile function and ventricular arrhythmias, which may result in sudden death. Abnormal intracellular  $\text{Ca}^{2+}$  handling is a likely mechanism contributing to decreased force development and has been associated with impaired SR  $\text{Ca}^{2+}$  storage function [3, 4, 15]. Luminal SR  $\text{Ca}^{2+}$  concentrations are determined by diastolic SR  $\text{Ca}^{2+}$  uptake through ATPases (SERCA2s) as well as diastolic  $\text{Ca}^{2+}$  leak via ryanodine receptors (RyR2s). Although the dynamic leak-load relationship may limit SR  $\text{Ca}^{2+}$  loss resulting from decreased SERCA2 function in HF [23], chronically increased RyR2 open probability may impair contractile performance [27] and may also lead to triggered arrhythmias [24].

Recently, novel pharmacological and genetic therapeutic strategies were developed which target abnormal intracellular  $\text{Ca}^{2+}$  cycling in HF and arrhythmias [5, 13, 22]. Among these, the 1,4-benzothiazepine compound K201 (also known as JTV-519 [9]) has shown beneficial effects in animals with HF [28, 31] or arrhythmias [26]. K201 has multiple sites of action in the heart. The most interesting action of K201 is the ability to induce a conformational change in RYR reducing its open probability [30]. Other actions of K201 are the inhibition of SERCA [14] and several sarcolemmal ion-channels [10]. These different actions were demonstrated to depend on the concentration of K201 and concentrations up to 1  $\mu\text{M}$  K201 are used to ensure RYR-mediated effects [12].

To investigate the efficacy of K201 to improve human cardiac muscle function, intact muscle preparations from terminally failing human hearts were used, and the force frequency response (FFR) and post-rest potentiation (PRP) protocols were applied to study SR-dependent muscle performance [18]. In particular, PRP has been shown to correlate with SR  $\text{Ca}^{2+}$  content [19]. Our data identify potentially beneficial K201 effects on contractile function in terminally failing human hearts.

## Methods

### Muscle preparation

Human ventricular muscle strips were dissected from freshly explanted hearts of 14 end-stage heart failure patients undergoing cardiac transplantation as a result of

ischemic or dilated cardiomyopathy (12 men and 2 women, average age  $42.2 \pm 4.2$  years). Unfortunately, the analysis of non-failing myocardium was not possible due to the lack of available tissue. Detailed patient characteristics are provided in the online supplement. The investigation conforms to the principles outlined in the Declaration of Helsinki. The study was approved by the institutional ethics committee, and all patients provided written informed consent for the use of cardiac tissue samples. Hearts were transported in a Krebs–Henseleit buffer (KHB) with 2,3-butanedione monoxime as cardioplegic solution [8]. Intact trabeculae were carefully microdissected from the left ventricle and fixed between a force transducer (Scientific Instruments) and a hook connected to a micro-manipulator for length adjustment. Only trabeculae with a diameter of 0.5 mm or less were used for experiments. Mean dimensions (mm) were as follows: diameter 1  $0.40 \pm 0.01$ ; diameter 2  $0.36 \pm 0.01$ ; length  $2.2 \pm 0.1$ . The distribution of the muscle diameter was not different between the analysed groups. After wash-out of the cardioplegic solution, muscle preparations were superfused with Krebs–Henseleit solution (containing in mmol/L: 137 NaCl, 5.4 KCl, 1.2  $\text{MgSO}_4$ , 1.2  $\text{NaH}_2\text{PO}_4$ , 20 HEPES, 10 glucose, 0.25  $\text{CaCl}_2$ ; pH adjusted to 7.4 with NaOH) and electrically stimulated (baseline 1 Hz, amplitude 3–5 V; stimulator Scientific Instruments type STIM2). Force measurements were carried out at 37°C and either at 1.25 or 5 mmol/L  $[\text{Ca}^{2+}]_o$ . After 60 min of equilibration, the muscles were stretched gradually to the length at which maximum steady-state twitch force was reached ( $L_{\text{max}}$ ).

### Experimental protocol

K201 was a gift from Aetas Pharma Ltd. (Japan). The drug was dissolved in DMSO (99.7%, Sigma-Aldrich, Taufkirchen, Germany) to achieve a nominal stock concentration of 1 mmol/L. To determine the effect of K201 on basal contractile performance, K201 was added to the circulating KHB solution in different concentrations (0.1, 0.3, 1, 3, 10, 30  $\mu\text{M}$ ) and compared to the control group treated with the drug carrier (DMSO, same vol% dilution corresponding to each K201 step). The developed tension ( $T_{\text{dev}}$ ), diastolic tension ( $T_{\text{dia}}$ ), time to peak (TTP) and time to 50 and 90% relaxation ( $\text{RT}_{50}$  and  $\text{RT}_{90}$ ) were recorded online using LabView (National instruments).

In all further experiments (Fig. 1), K201 was added to the solution at least 60 min before assessment of muscles strip function. Force-frequency relationship and PRP were analysed in K201 (0.3 or 1  $\mu\text{M}$ ) treated preparations. The FFR was examined at a range of 0.25–3 Hz and normalised to 0.25 Hz. Force recording of isometric tension was obtained at steady-state conditions at each frequency step. PRP was examined using rest intervals of 1–120 s (1, 2, 4,

8, 16, 30, 60, 120 s). The tension developed on the first twitch after rest was divided by the mean developed tension of the last ten beats before rest.

To provoke an increase in SR  $\text{Ca}^{2+}$  leak and diastolic tension extracellular calcium ( $[\text{Ca}^{2+}]_e$ ) was elevated stepwise from 1.25 to 5 mmol/L [1, 2.] Upon steady state, PRP protocol was conducted as described above to ascertain equal starting conditions in all groups. Then the muscles were randomised to treatment with either K201 at 0.3 or 1  $\mu\text{M}$  or DMSO and left contracting for 2 h. After 2 h the PRP was repeated again.

To examine the influence of calcium on diastolic tension muscles were treated with 0.3  $\mu\text{M}$  K201 or DMSO at the start of the experiment and stretched to  $L_{\text{max}}$  at 1.0 mmol/L  $[\text{Ca}^{2+}]_e$ . Upon steady state the  $[\text{Ca}^{2+}]_e$  was increased stepwise (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0) to 5 mmol/L. Contractile parameters were analysed after every step. Finally, FFR was measured in these muscles strips with 5 mmol/L  $[\text{Ca}^{2+}]_e$ .

Mean developed tension of failing human myocardial muscle strips at 1 Hz was  $12.2 \pm 2.1 \text{ mN/mm}^2$ .

## Calculation and statistics

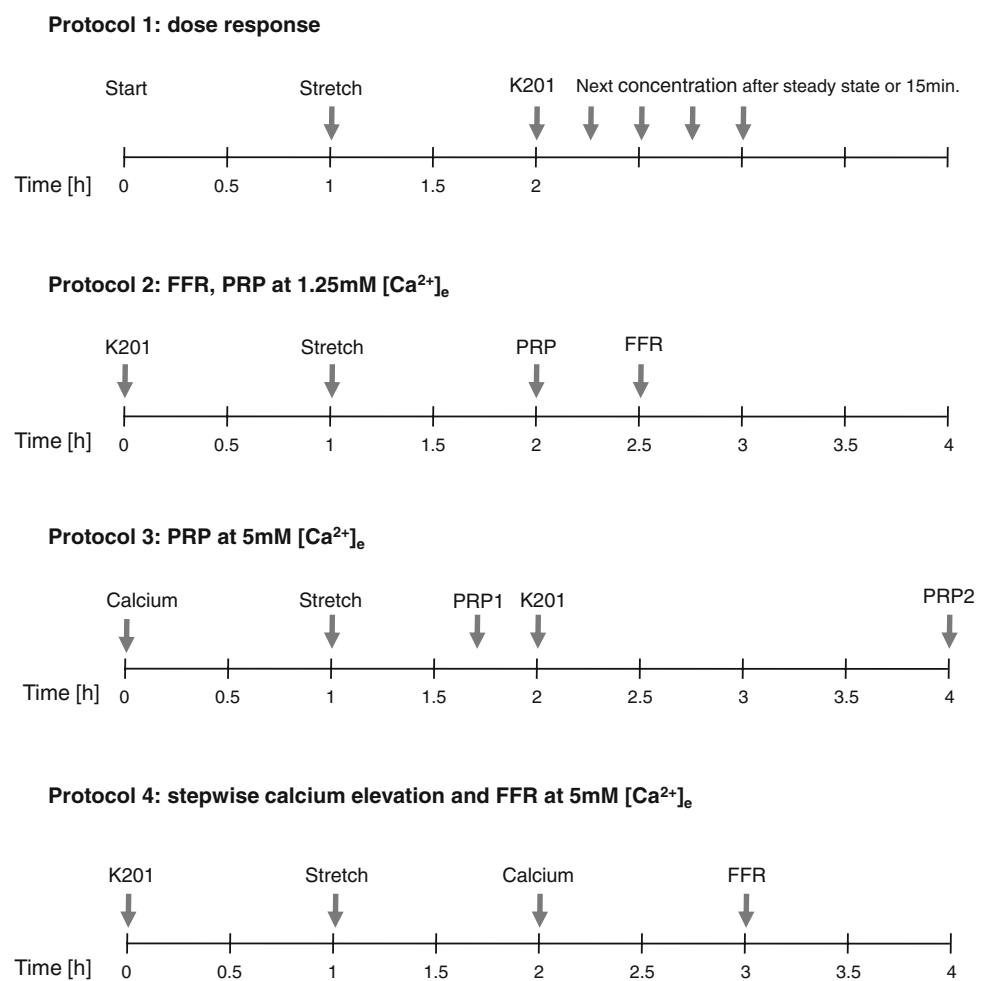
Tension measurements were normalised to the cross-sectional area for each preparation, calculated assuming an elliptical cross section using the formula cross-sectional area =  $D_1/2 \times D_2/2 \times \pi$ , with  $D_1$  and  $D_2$  representing width and thickness, and expressed as tension. Determined parameters were expressed as the ratio of the respective baseline parameter. Contractile parameters at different K201 concentrations were analysed using two-way ANOVA or paired Students  $t$  test where appropriate, with values of  $P < 0.05$  considered statistically significant.

## Results

### Concentration-dependent response to K201

Compared to control ( $n = 6$ ), developed tension ( $T_{\text{dev}}$ ) remained unchanged until 0.3  $\mu\text{M}$  K201 and declined at a concentration of 1  $\mu\text{M}$  (1  $\mu\text{M}$ :  $9.8 \pm 2.5\%$  of control,

**Fig. 1** Schematic diagram of the experimental protocols. *K201* Addition of K201, *Stretch* stretch of the muscles strip to  $L_{\text{max}}$ , *FFR* measurement of the force frequency relationship, *PRP* measurement of the post rest potentiation, *Calcium* increase of the  $[\text{Ca}^{2+}]_e$  from 1.25 mmol/l stepwise to 5.0 mmol/l



$P < 0.05$ ,  $n = 6$ , Fig. 2). Higher doses of K201 lead to a further reduction of  $T_{dev}$ . At 30  $\mu\text{M}$  the developed tension was reduced to only  $23 \pm 5\%$  ( $P < 0.001$ ) of control. The half-maximal inhibitory concentration ( $IC_{50}$ ) of K201 on the developed tension was 8.7  $\mu\text{M}$  (Supplementary figure 1).

#### Effects of K201 on contractile performance at low $[\text{Ca}^{2+}]_e$ in failing human hearts

Isolated ventricular muscle preparations from terminally failing human hearts did not show an acutely improved contractile function following K201 at 0.3  $\mu\text{M}$  and clearly K201 may be acutely negatively inotropic at high concentrations (Fig. 2; Supplementary figure 1). However, K201 has a profound effect on post rest potentiation even at concentrations of 0.3  $\mu\text{M}$ , where contractility is largely preserved (Fig. 3a). In human failing cardiac muscle strips we typically observe a robust post rest potentiation (Fig. 3b). Up to 16 s the increase in post rest potentiation was similar between K201 or control treated muscle strips (increase from 0 to 16 s: control:  $93 \pm 17\%$ ; 0.3  $\mu\text{M}$  K201:  $87 \pm 12\%$ ; 1  $\mu\text{M}$  K201:  $94 \pm 11\%$ , each  $P < 0.01$ , Fig. 3b). At longer rest intervals, a further increase in post rest potentiation was visible in the K201 treated muscles

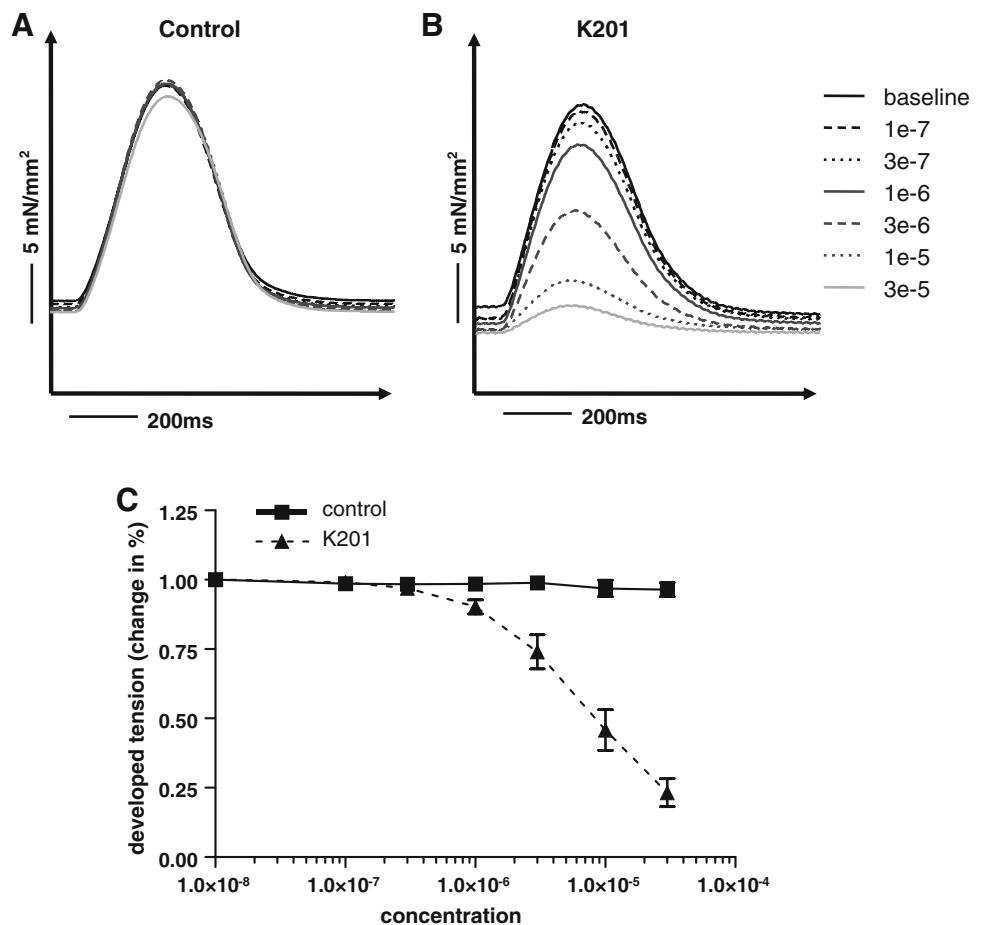
strips (increase from 16 to 120 s: 0.3  $\mu\text{M}$  K201:  $59 \pm 27\%$ ; 1  $\mu\text{M}$  K201:  $62 \pm 34\%$ , each  $P < 0.05$ ), but not in the control group (increase from 16 to 120 s:  $7 \pm 14\%$ ,  $P = 0.61$ , Fig. 3c).

Additionally, we observed a frequency-dependent significant increase in diastolic tension and a blunted increase in systolic tension in failing human myocardium. However, both diastolic and systolic FFR were not significantly altered by either K201 treatment (0.3 or 1  $\mu\text{M}$ ) compared to control (Supplementary figure 2). These results are in agreement with earlier data from failing human myocardium demonstrating that the phenotype of increased diastolic tension in combination with a blunted FFR is associated with decreased SERCA2 and increased NCX function, further contributing to depletion of intracellular  $\text{Ca}^{2+}$  stores through competitive mechanisms and explaining the relatively long PRP needed to observe beneficial efficacy of K201 earlier.

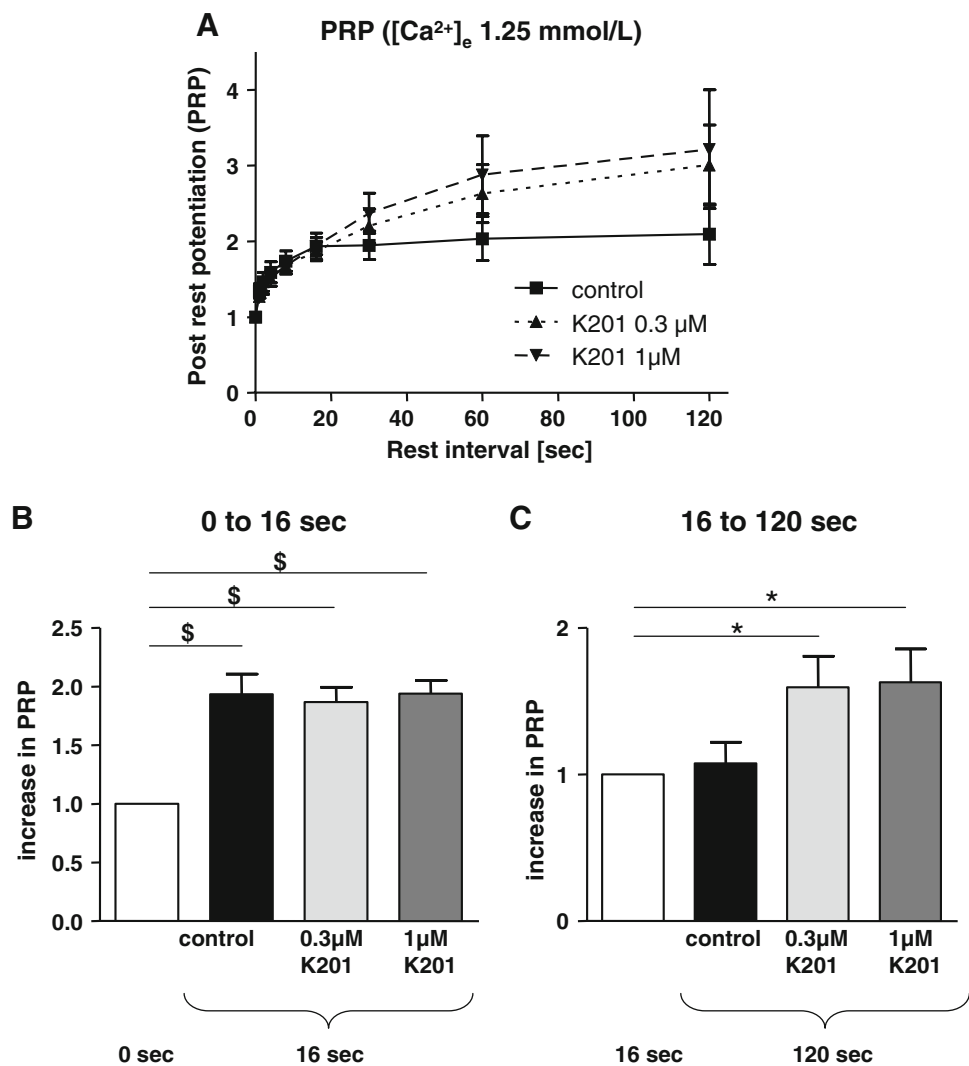
#### Influence of K201 treatment on PRP behaviour at elevated $[\text{Ca}^{2+}]_e$ in human failing hearts

Since our initial data indicated that K201 effects on PRP behaviour required relatively long rest intervals under

**Fig. 2** Example of recorded contraction before and following addition of vehicle control (DMSO, **a**) or K201 (**b**) in concentrations from 100 nM to 30  $\mu\text{M}$  at 1 Hz. **c** Effect on developed tension in human failing muscles strips [K201 dotted line vs. control (DMSO) solid line, each  $n = 6$ ]



**Fig. 3** Effect of 0.3  $\mu\text{M}$  (dotted line,  $n = 6$ ) and 1  $\mu\text{M}$  K201 (dashed line,  $n = 6$ ) and control (solid line,  $n = 6$ ) on PRP at 1.25 mmol/L  $[\text{Ca}^{2+}]_e$  (a). Increase of post rest potentiation at 16 s normalised to 0 s (b) and at 120 s normalised to 16 s (c) in control (black bar) and 0.3  $\mu\text{M}$  (light gray bar) or 1  $\mu\text{M}$  (dark gray bar) K201. \* $P < 0.05$ ,  $^{\$}P < 0.01$



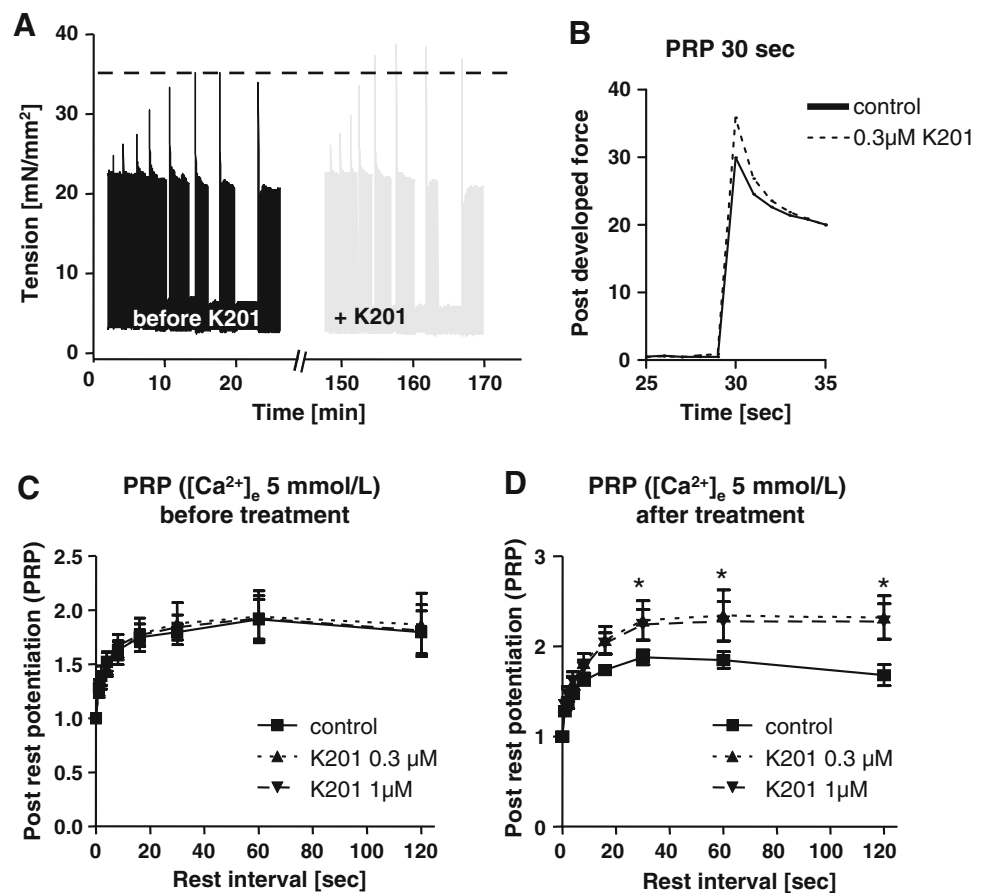
relatively  $\text{Ca}^{2+}$  deprived conditions, we investigated PRP before and after prolonged (2 h) treatment with 0.3 or 1  $\mu\text{M}$  K201 (Fig. 4a, b). Before treatment PRP was not different between the experimental groups (Fig. 4c). However, PRP was significantly increased by 38 and 35% using 0.3 or 1  $\mu\text{M}$  K201, respectively (normalised increase after a rest interval of 120 s:  $1.68 \pm 0.12$  in the control group and  $2.32 \pm 0.24$  and  $2.28 \pm 0.20$  in the K201 group with a concentration of 0.3 and 1  $\mu\text{M}$ , respectively  $P < 0.05$ , Fig. 4d).

#### Effect of K201 on contractile performance during elevation of $[\text{Ca}^{2+}]_e$ in human failing hearts

Since our initial data indicated that K201 effects on PRP behaviour required relatively long rest intervals under relatively  $\text{Ca}^{2+}$  deprived conditions, we investigated failing ventricular preparations at supra-physiological  $[\text{Ca}^{2+}]_e$  concentrations. Above normal  $[\text{Ca}^{2+}]_e$  can be expected to

lead to a relative increase in SR intraluminal  $\text{Ca}^{2+}$  concentrations [20]; however, the pre-existing competition between SERCA2 and NCX can be expected to partially extrude the additional and potentially inotropic  $\text{Ca}^{2+}$  to the extracellular side. Therefore, K201 by inhibiting SR  $\text{Ca}^{2+}$  leak may improve SR  $\text{Ca}^{2+}$  load independently from pre-existing SERCA2 and NCX mechanisms. As expected, increased  $[\text{Ca}^{2+}]_e$  (5 mmol/L) resulted in a significant increase in diastolic tension (Fig. 5a) of  $+25.7 \pm 5.9\%$  (relative to baseline conditions at 1 mmol/L;  $P < 0.05$ , each  $n = 7$ ; Fig. 4c). However, K201 treatment (0.3  $\mu\text{M}$ ) abolished the increase in diastolic tension ( $n = 7$ ,  $P < 0.001$ , Fig. 5b, c). Additionally, following the same K201 treatment developed tension ( $T_{\text{dev}}$ ) was significantly increased by  $+27 \pm 8\%$  ( $P < 0.05$ ; Fig. 5c) at 5 mmol/L  $[\text{Ca}^{2+}]_e$ . In agreement with earlier reports [16], myocardial relaxation was significantly prolonged at higher  $\text{Ca}^{2+}$  concentrations in control preparations ( $\text{RT}_{90}$  1.0 vs. 5.0 mmol/L  $[\text{Ca}^{2+}]_e$ :  $+19 \pm 7\%$ ,  $P < 0.05$ ). However,

**Fig. 4** Representative examples of a PRP (a) before (black) and 2 h after (grey) treatment with K201 at 5 mmol/L  $[Ca^{2+}]_e$ . The dashed line shows the maximal increase of post rest potentiation of the untreated muscles strip. Example of post rest contractile performance (b) after 30 s rest interval in control (black) and 0.3  $\mu$ M K201 (dashed line). Comparison of the effect of 0.3  $\mu$ M (dotted line,  $n = 7$ ) or 1  $\mu$ M K201 (dashed line,  $n = 7$ ) and control (solid line,  $n = 7$ ) on PRP at 5 mmol/L  $[Ca^{2+}]_e$  before (c) and 2 h after (d) treatment with K201 or control. \* $P < 0.05$ ,  $^{\$}P < 0.05$



K201 treatment prevented the increase in relaxation time ( $RT_{90}$  1.0 vs. 5.0 mmol/L  $[Ca^{2+}]_e$ :  $+14 \pm 10\%$ , not significant). Thus, our data indicate that K201 exerts beneficial effects including myocardial contraction and relaxation in failing human myocardium.

#### Influence of K201 on the FFR at elevated $[Ca^{2+}]_e$ in human failing hearts

Increasing the stimulation frequency in muscles strips with elevated  $[Ca^{2+}]_e$  leads to a frequency-dependent decline in developed tension and an increase in diastolic tension. Taking into account that the contractile performance at 1 Hz was already different in the K201-treated group compared to control, the frequency-dependent changes in developed and diastolic tension were normalised to the tension at 1 Hz and 1.0 mmol/L  $[Ca^{2+}]_e$  (Fig. 6a, c). The contractile improvement by K201 was preserved also at higher frequencies (Fig. 6b, d). The developed tension was higher in the K201-treated muscles strips at the stimulation frequencies from 1 to 3 Hz (K201 vs. control: 1 Hz:  $2.99 \pm 0.25$  vs.  $2.35 \pm 0.11$ ; 2 Hz:  $1.50 \pm 0.23$  vs.  $0.89 \pm 0.14$ ; 3 Hz:  $0.65 \pm 0.09$  vs.  $0.39 \pm 0.06$ ; each  $n = 7$  and  $P < 0.05$ , Fig. 6b). Also the diastolic tension

was lower throughout the whole range of the FFR (K201 vs. control: 1 Hz:  $1.01 \pm 0.03$  vs.  $1.25 \pm 0.06$ ; 2 Hz:  $1.69 \pm 0.35$  vs.  $3.07 \pm 0.69$ ; 3 Hz:  $2.13 \pm 0.47$  vs.  $3.75 \pm 0.75$ ; each  $n = 7$  and  $P < 0.05$ , Fig. 6d).

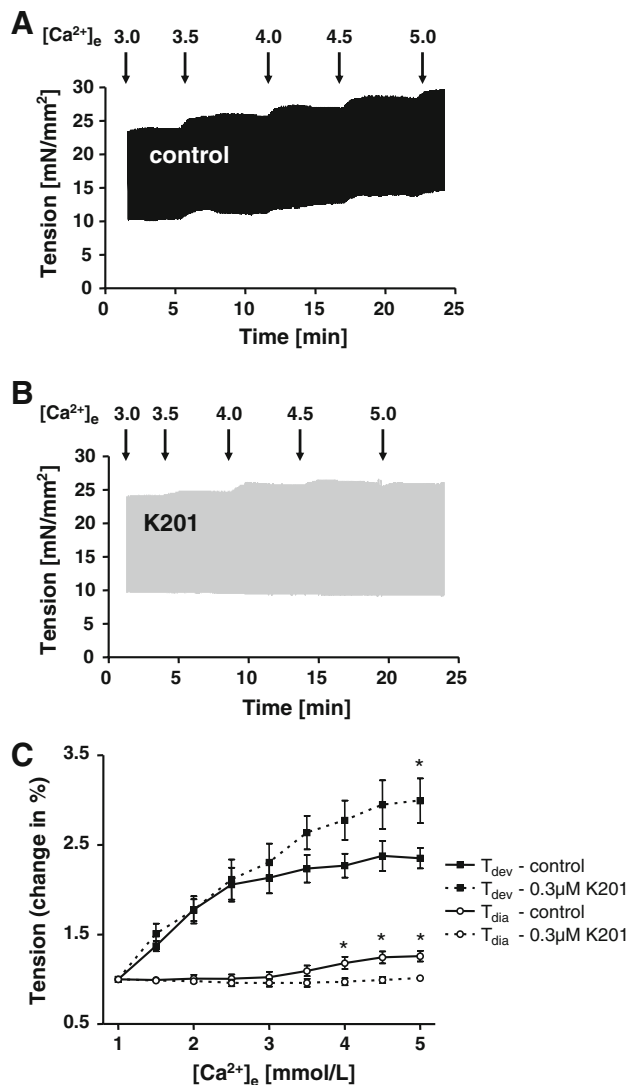
#### Discussion

Our data suggest that the 1,4-benzothiazepine K201 improves both diastolic and systolic contractile function of failing human myocardium. The following observations support these conclusions: (1) PRP ( $[Ca^{2+}]_e$  1.25 mM) was improved by K201 at longer post-rest intervals; (2) K201 ( $[Ca^{2+}]_e$  5 mM) improved PRP at shorter post-rest intervals; (3) K201 prevented diastolic dysfunction and improved systolic function under  $Ca^{2+}$  overload conditions; (4) the beneficial effects on systolic and diastolic function were maintained throughout the physiological frequency range of the human heart.

Importantly, systolic improvement is confined to low concentrations of K201 as higher concentrations are clearly negatively inotropic.

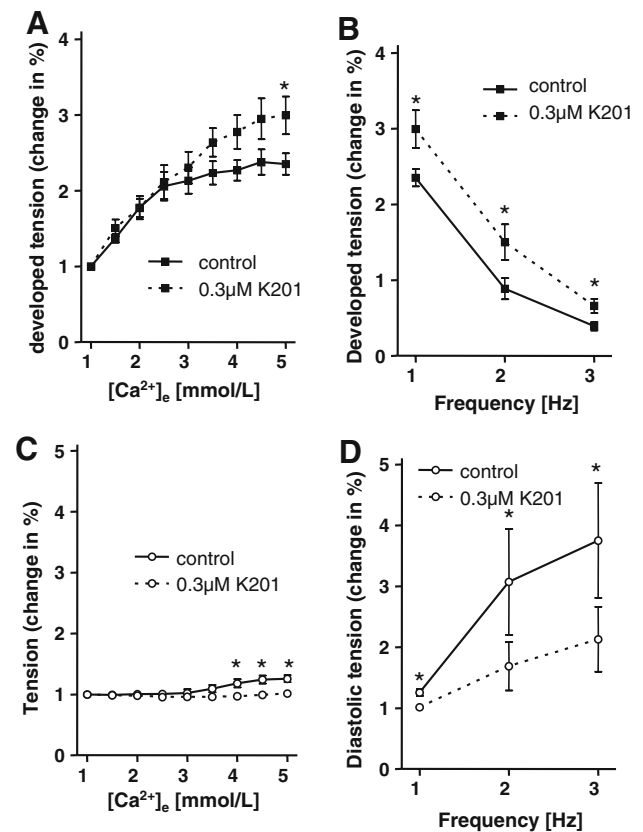
Apart from the potentially therapeutic effects of K201 on RyR2 dysfunction and intracellular  $Ca^{2+}$  leak [9, 11, 12],





**Fig. 5** Original recordings from two experiments during the increase of [Ca<sup>2+</sup>]<sub>e</sub> from 3.0 to 5.0 mmol/L from control (a) or with 0.3 μM K201 (b) muscle strip experiments. c Effect of [Ca<sup>2+</sup>]<sub>e</sub> on the diastolic (open symbols) and developed tension (filled symbols) of human failing muscle strips treated with either 0.3 μM K201 (dotted line, *n* = 7) or control (solid line, *n* = 7). Tension is expressed relative to tension at [Ca<sup>2+</sup>]<sub>e</sub> of 1 mmol/L, \**P* < 0.05

K201 has documented off-target effects at high micromolar concentrations. Therefore, the negative inotropic effect on developed tension in failing human myocardial preparations may be attributed to inhibitory actions of micromolar K201 concentrations on L-type Ca<sup>2+</sup> currents and SERCA [10, 14]. However, at nanomolar concentrations of K201 (0.3 μM), no adverse effects on the contractile performance of failing human myocardium were observed. Additionally, at low physiological [Ca<sup>2+</sup>]<sub>e</sub> contractile function was not affected by K201 treatment in failing muscle preparation. This finding is in agreement with studies showing that SR Ca<sup>2+</sup> leak depends on sufficient SR Ca<sup>2+</sup> load [29] and that



**Fig. 6** After elevation of the [Ca<sup>2+</sup>]<sub>e</sub> to 5.0 mmol/L (a, c) the frequency was increased from 1 to 2 and 3 Hz (b, d). The frequency-dependent changes of human failing muscles strips treated with either 0.3 μM K201 (dotted line, *n* = 7) or control (solid line, *n* = 7) in developed (b) and diastolic tension (d) were normalised to the tension at 1.0 mmol/L [Ca<sup>2+</sup>]<sub>e</sub> and 1 Hz taking into account, that the contractile performance at 5.0 mmol/L [Ca<sup>2+</sup>]<sub>e</sub> is already different in the K201-treated muscle strips compared to controls. *P* < 0.05

extracellular Ca<sup>2+</sup>-depletion leads to reduced SR Ca<sup>2+</sup> load and reduced SR Ca<sup>2+</sup> leak [23]. Thus, our data under low [Ca<sup>2+</sup>]<sub>e</sub> conditions agree with the hypothesis that K201 targets SR Ca<sup>2+</sup> leak but, importantly, does not block physiological SR Ca<sup>2+</sup> release and therefore exerts no beneficial or adverse effects. Additionally, K201 showed significant beneficial effects on PRP at longer post-rest intervals which have been associated with increased SR Ca<sup>2+</sup> load [20]. Again, these data support that K201 effects depend on the existence of significant SR Ca<sup>2+</sup> leak in human failing muscle strips due to longer SR Ca<sup>2+</sup> load periods.

Kimura et al. [10] showed that the half-maximal inhibitory concentration (IC<sub>50</sub>) of K201 on plasma membrane ion currents is 5 μM. Loughrey et al. [14] showed that K201 in a concentration of 3 μM significantly reduced SERCA activity. However, we have not observed any adverse changes on FFR behaviour throughout the entire physiological frequency range of the human heart with

K201 at 0.3  $\mu\text{M}$ ; thus our results may be explained by a up to a magnitude order lower K201 concentrations.

Indeed, experiments with human cardiac muscle preparations have been established as a solid model of cardiac excitation–contraction-coupling. For example, it has been shown that the FFR in isolated muscle preparations resembles the FFR in patients with HF [6], and that intracellular  $\text{Ca}^{2+}$  cycling is depressed in muscle preparations following post-rest activation [20]. Accordingly, we have used increased  $[\text{Ca}^{2+}]_e$  as a model of increased intracellular  $\text{Ca}^{2+}$  load in accordance with previously established protocols [1, 2] to characterise potential beneficial effects of K201 under conditions of increased SR  $\text{Ca}^{2+}$  leak as shown earlier [14]. As we did not measure  $\text{Ca}^{2+}$  directly, our study relies on previous observations showing that SR  $\text{Ca}^{2+}$  leak is a function of SR  $\text{Ca}^{2+}$  load [29] and that SR  $\text{Ca}^{2+}$  concentration in intact cardiac myocytes depends directly on intracellular  $\text{Ca}^{2+}$  concentration [23]. Importantly, an increase in intracellular  $\text{Ca}^{2+}$  concentration was achieved by increasing  $[\text{Ca}^{2+}]_e$  as reported earlier in non-human material [1] and was reported to be associated with multiple spontaneous SR  $\text{Ca}^{2+}$  release events [14].

Indeed, following K201 treatment, failing human muscle strip preparations showed improved PRP behaviour at shorter post-rest intervals under high  $[\text{Ca}^{2+}]_e$  conditions. These results are in line with previously published animal data [11, 27] and demonstrate beneficial effects of nanomolar concentrations of K201 (0.3  $\mu\text{M}$ ). Our results confirm earlier animal studies by Kohno et al. [11] who have found efficacy of K201 against intracellular  $\text{Ca}^{2+}$  leak at the same concentration.

A limitation of our study is the lack of direct  $\text{Ca}^{2+}$  measurements. Although this was achieved technically in the past, in our hands it is clearly not possible to obtain these measurements at physiological temperature and stimulus rates (whereas the data presented here was measured at 37°C under near physiological conditions).

A significant finding is that K201 improved both diastolic and developed tension throughout the physiological frequency range of the human heart. Indeed, altered FFR is an important pathophysiological mechanism of reduced cardiac performance and altered stress adaptation in human HF [18, 21]. The combined beneficial effects of K201 on diastolic function and the FFR behaviour in failing human myocardium indicate potentially beneficial effects of 1,4-benzothiazepines which are distinct from existing pharmacological strategies.

Previously the mechanism of K201 on RYR2 stabilisation could be further deciphered: Yamamoto et al. [32] found that the K201-binding site on RYR2 is the domain (2114–2149). Interdomain interaction of RyR2 becomes loose in failing hearts, resulting in SR  $\text{Ca}$  leak [17, 25].

Addition of K201 to the destabilised RyR2 restores a stable configuration, i.e. inter-domain interaction, as in RyR2 from non-failing myocardium [32]. Rebinding of FKBP12.6 to RyR2 in failing SR seems not to be required to induce the K201 effects on RYR2 [7, 17], but K201 facilitates rebinding of FKBP12.6 in some cases [26, 30], suggesting that K201 may act primarily by modifying domain–domain interaction, but less via FKBP12.6.

We observed a negative inotropic effects at higher concentrations of K201 suggesting a detrimental effect on cardiac performance in failing human hearts. Although this finding does not necessarily imply an unfavourable effect in heart failure (i.e.  $\beta$ -blocker therapy paradoxically improves cardiac function in heart failure), we suggest K201 should be used at concentrations lower than 1  $\mu\text{M}$  when initially examined in humans. Our findings suggest that at such low concentrations there is already a profound effect on calcium cycling in human myocytes without negative inotropy. This gives support to the concept that the antiarrhythmic effects evident in multiple animal models at these lower K201 concentrations will be also present in humans.

In summary, this study demonstrates that K201 improved some aspects of the contractile performance in the human failing myocardium at lower concentrations (0.3  $\mu\text{M}$ ) consistent with reduced SR calcium leak. Therefore K201 may, in addition to previously reported anti-arrhythmic effects, improve contractile performance in the failing human heart.

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**Conflict of interest statement** None.

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