

K201 (JTV-519) alters the spatiotemporal properties of diastolic Ca^{2+} release and the associated diastolic contraction during β -adrenergic stimulation in rat ventricular cardiomyocytes

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Abstract K201 has previously been shown to reduce diastolic contractions in vivo during β -adrenergic stimulation and elevated extracellular calcium concentration ($[\text{Ca}^{2+}]_o$). The present study characterised the effect of K201 on electrically stimulated and spontaneous diastolic sarcoplasmic reticulum (SR)-mediated Ca^{2+} release and contractile events in isolated rat cardiomyocytes during β -adrenergic stimulation and elevated $[\text{Ca}^{2+}]_o$. Parallel experiments using confocal microscopy examined spontaneous diastolic Ca^{2+} release events at an enhanced spatiotemporal resolution. 1.0 $\mu\text{mol/L}$ K201 in the presence of 150 nmol/L isoproterenol (ISO) and 4.75 mmol/L $[\text{Ca}^{2+}]_o$ significantly decreased the amplitude of diastolic

contractions to $\sim 16\%$ of control levels. The stimulated free Ca^{2+} transient amplitude was significantly reduced, but stimulated cell shortening was not significantly altered. When intracellular buffering was taken into account, K201 led to an increase in action potential-induced SR Ca^{2+} release. Myofilament sensitivity to Ca^{2+} was not changed by K201. Confocal microscopy revealed diastolic events composed of multiple Ca^{2+} waves (2–3) originating at various points along the cardiomyocyte length during each diastolic period. 1.0 $\mu\text{mol/L}$ K201 significantly reduced the (a) frequency of diastolic events and (b) initiation points/diastolic interval in the remaining diastolic events to 61% and 71% of control levels respectively. 1.0 $\mu\text{mol/L}$ K201 can reduce the probability of spontaneous diastolic Ca^{2+} release and their associated contractions which may limit the propensity for the contractile dysfunction observed in vivo.

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Sarcoplasmic reticulum · Calcium waves

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular calcium concentration
$[\text{Ca}^{2+}]_o$	Extracellular calcium concentration
DAD	Delayed after depolarisations
DD	Diastolic dysfunction
F/F_0	Quiescent fluorescence
I_{Ca}	L-type Ca^{2+} current
KH	Krebs–Henseleit solution
NCX	Sodium–calcium exchanger
NE	Nor-epinephrine
RCL	Resting cell length
RyR2	Ryanodine receptor
SERCA	Sarcoplasmic/endoplasmic Ca^{2+} ATPase pump
SR	Sarcoplasmic reticulum

Introduction

Sarcoplasmic reticulum (SR)-mediated Ca^{2+} handling (which includes Ca^{2+} uptake via the sarcoplasmic/endoplasmic Ca^{2+} ATPase pump, SERCA, and Ca^{2+} release via the ryanodine receptor, RyR2) is altered in almost all models of heart failure and may lead to abnormal myocardial relaxation and filling during diastole [5, 6, 15, 22]. While many studies have correlated the consequences of altered SERCA-mediated Ca^{2+} uptake and diastolic dysfunction (DD) [1, 20], few have investigated the consequences of abnormal diastolic SR Ca^{2+} release on whole heart contractile function. To address this issue, a recent study by the authors demonstrated that while in vivo administration of a β -adrenergic agonist or an elevated extracellular calcium concentration ($[\text{Ca}^{2+}]_o$) separately did not produce any significant alteration of diastolic function in rat hearts, co-administration of a β -adrenergic agonist and an elevated $[\text{Ca}^{2+}]_o$ led to an acute onset of DD [13]. A β -adrenergic agonist was used in this in vivo model to stimulate a PKA-mediated increase in SR Ca^{2+} load. When combined with an elevated $[\text{Ca}^{2+}]_o$, DD manifest as diastolic contractions, a raised end-diastolic pressure (EDP) but no alteration of ejection fraction [13].

K201 (JTV-519), a 1,4 benzothiazepine derivative structurally distinct from diltiazem, was originally discovered when screening compounds to prevent sudden cardiac cell death in a rat Langendorff model of myofibrillar over-contraction [12]. The drug has been suggested to improve contractile function in heart failure via a sole inhibitory effect on diastolic RyR2-mediated SR Ca^{2+} release [27, 29]. However, it is clear from the literature that K201 exhibits effects on additional cellular targets in a species-specific manner [3]. In guinea pig cardiomyocytes, K201 inhibits sodium (I_{Na}) [16] and potassium currents (I_{K1} , I_{Kr}) [16, 17] and prolongs action potential duration [17], yet the effect on these targets has not been determined in alternative species. Investigations of the effect on L-type calcium current (I_{Ca}) have yielded contrasting results with inhibition in the rat and guinea pig [11, 14], whilst no effect in the rabbit [18]. Inhibition of SERCA activity by K201 in rabbit cardiomyocytes has been demonstrated previously, but the effect in rat is unknown [18]. In the above rat model of DD, K201 limits diastolic contractures and reduces EDP [14] in vivo; however, the cellular mechanisms underlying these phenomena are unknown. It is possible that the observed benefit of K201 in vivo could originate from the drugs ability to (a) inhibit abnormal diastolic Ca^{2+} release events leading to improved contractile performance or (b) alteration of myofilament Ca^{2+} sensitivity. To test these hypotheses and given K201's species specificity, this study was designed to characterise the effect of K201 on: (a) the stimulated Ca^{2+} transient and cell shortening,

(b) spontaneous Ca^{2+} release and the corresponding spontaneous contractile event, (c) myofilament sensitivity to Ca^{2+} and (d) SR function, in isolated rat cardiomyocytes exposed to an elevated $[\text{Ca}^{2+}]_o$ and a commonly used β -adrenergic agonist–isoproterenol (ISO).

Materials and methods

Adult rat ventricular cardiomyocyte isolation

Adult male Wistar rats (200–300 g, 8–9 weeks in age, $n = 23$) were euthanased using a schedule one procedure in accordance with the UK Animals (Scientific Procedures) Act 1986. Hearts were removed and perfused retrogradely at 5 mL min^{-1} (37°C) with a modified isolation Krebs-Henseleit (KH) solution for 4 min. The composition of the isolation KH was (in mmol/L): NaCl (120), KCl (5.4), HEPES (20), $\text{Na H}_2\text{PO}_4$ (0.52), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (3.5), taurine (20), creatine (10), glucose (11.1), pH 7.4 with NaOH. This was followed by perfusion with KH containing 1 mg mL^{-1} collagenase (type I, Worthington Biochemical, Lakewood, NJ, USA), and 0.1 mg mL^{-1} protease (type XIV, Sigma-Aldrich, UK). After ~ 6.0 min, enzyme was removed and the heart perfused with nominally Ca^{2+} -free isolation KH solution containing 1% BSA (Sigma-Aldrich, UK) for a further 6.0 min. The left ventricular free wall was then cut into strips and mixed to yield a single cell suspension. Cells were maintained in either Ca^{2+} -free isolation KH solution or $1.0 \text{ mmol/L Ca}^{2+}$ (via stepwise increments) until use.

Field stimulation of intact cardiomyocytes with simultaneous whole cell epi-fluorescence and shortening measurements

Intact cardiomyocytes in modified isolation KH solution (above) were loaded with a Ca^{2+} sensitive fluorophore ($5.0 \mu\text{mol/L}$ Fura-4F AM, Invitrogen, UK) by incubation for ~ 10 min. The incubation medium was removed and the cells re-suspended in a second modified KH solution with the following composition (in mmol/L): NaCl (140), KCl (4.0), MgCl_2 (1.0), HEPES (5.0), glucose (11.1), pH 7.4 with NaOH with $1.25 \text{ mmol/L } [\text{Ca}^{2+}]$ added. Cells were incubated for a further 30 min to ensure complete de-esterification. Cardiomyocytes were allowed to settle on a coverslip, placed on a bath (Cell Microcontrols, Norfolk, VA, USA) and super-fused with the same modified KH solution at 37°C . Cells were field stimulated with 2 ms duration voltage pulses delivered through parallel platinum wires (stimulation voltage set to 1.5 times the threshold). K201 (a gift from Aetas Pharma Co. Ltd., Japan) was prepared at a stock concentration of 1.0 mmol/L in 10% DMSO; the final concentration of K201 in the perfusate

was 1.0 $\mu\text{mol/L}$. A parallel set of vehicle (DMSO) time control experiments were performed; all data obtained in K201 were normalised to these timed controls. The Fura-4F fluorescence (340 and 380 nm excitation; $R_{340/380\text{nm}}$) was measured using a spinning wheel spectrophotometer (Cairn Research Ltd., UK; sampling rate of 500 Hz) whilst cellular shortening was measured using a video edge detection system (IonOptix, Milton, MA, USA; sampling rate of 200 Hz). Data were analysed offline. Fura-4F fluorescence ratio was converted to intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) as previously described [21]. Mean $[\text{Ca}^{2+}]_i$ and cell shortening signals were obtained by averaging 12 steady state transients (Origin; Online resource I). The mean minimum $[\text{Ca}^{2+}]_i$ was measured from a single point at which $[\text{Ca}^{2+}]_i$ was at its lowest during the diastolic period [see online resource I(b)]. Diastolic Ca^{2+} event amplitude, the degree of spontaneous Ca^{2+} release, was calculated from the minimum $[\text{Ca}^{2+}]_i$ to the peak diastolic $[\text{Ca}^{2+}]_i$ reached during the diastolic period. Diastolic Ca^{2+} events do not necessarily return to the minimum $[\text{Ca}^{2+}]_i$ before the next action-potential stimulation. The $[\text{Ca}^{2+}]_i$ immediately preceding the point of stimulation is therefore termed as the pre-stimulus $[\text{Ca}^{2+}]_i$ (pre-stim). Pre-stim $[\text{Ca}^{2+}]_i$ was subtracted from the stimulated $[\text{Ca}^{2+}]_i$ transient peak to determine stimulated Ca^{2+} transient amplitude. Shortening data were expressed as percentage of resting cell length (% RCL) where resting cell length was taken at the quiescent cell length prior to commencement of field stimulation. Cell shortening parameters were measured as described above for $[\text{Ca}^{2+}]_i$ and shown in Online resource Ib.

Myofilament Ca^{2+} sensitivity measurements in intact cells

Intact cardiomyocytes in modified KH solution were Fura-4F AM loaded and field stimulated as above. Fluorescence and cell shortening were assessed in three subsets of experiments during perfusion with KH solution containing: (a) a range of $[\text{Ca}^{2+}]_o$ (0.5–1.8 mmol/L), (b) 1.8 mmol/L $[\text{Ca}^{2+}]_o$ in the presence of K201 (0.3, 1.0 and 3.0 $\mu\text{mol/L}$) and (c) 1.8 mmol/L $[\text{Ca}^{2+}]_o$ in the presence of diltiazem (4.0 and 8.0 $\mu\text{mol/L}$, Sigma-Aldrich, UK). Data obtained in KH solution with a $[\text{Ca}^{2+}]_o$ of 1.8 mmol/L were obtained for each sub-set of experiments (to account for any variation between cell populations) and served as respective control groups. Fura-4F fluorescence was converted to $[\text{Ca}^{2+}]_i$ as above and cell shortening amplitudes were expressed as a percentage of the shortening amplitude at a $[\text{Ca}^{2+}]_o$ of 1.8 mmol/L. An additional set of experiments in permeabilised cells directly assessed the effect of K201 on the myofilaments (Online resource II).

Simultaneous field stimulation of intact cardiomyocytes with confocal imaging

Intact cardiomyocytes in a modified KH solution were loaded with Fluo-3AM and superfused with modified KH (composition as above). Confocal line-scan images of field stimulated cardiomyocytes were recorded using a Radiance 2000 confocal system (BioRad, UK). Fluo-3 was excited at 488 nm (Kr laser) and measured >515 nm using epifluorescence optics of an inverted microscope with a 60X/1.2 NA water-immersion objective lens. Fluorescence was acquired in line-scan mode at 2 ms line^{-1} ; pixel dimension was 0.3 μm (512 pixels/scan; zoom = 1.4) [2]. The scanning laser line was orientated parallel with the long axis of the cell and placed approximately equidistant between the outer edge of the cell and the nucleus/nuclei to ensure the nuclear area was not included in the scan line. Exact timing of electrical stimulation was marked in the confocal image by activating a light-emitting diode above the cell bath for 2 ms (i.e. duration of one line-scan), 8 ms before electrical stimulation. Fluorescence data were expressed as a ratio of the quiescent fluorescence (F/F_0). F/F_0 measurements and the number of initiation points/diastolic interval were calculated from 15 s periods of line scan trace.

Data analysis and statistical procedures

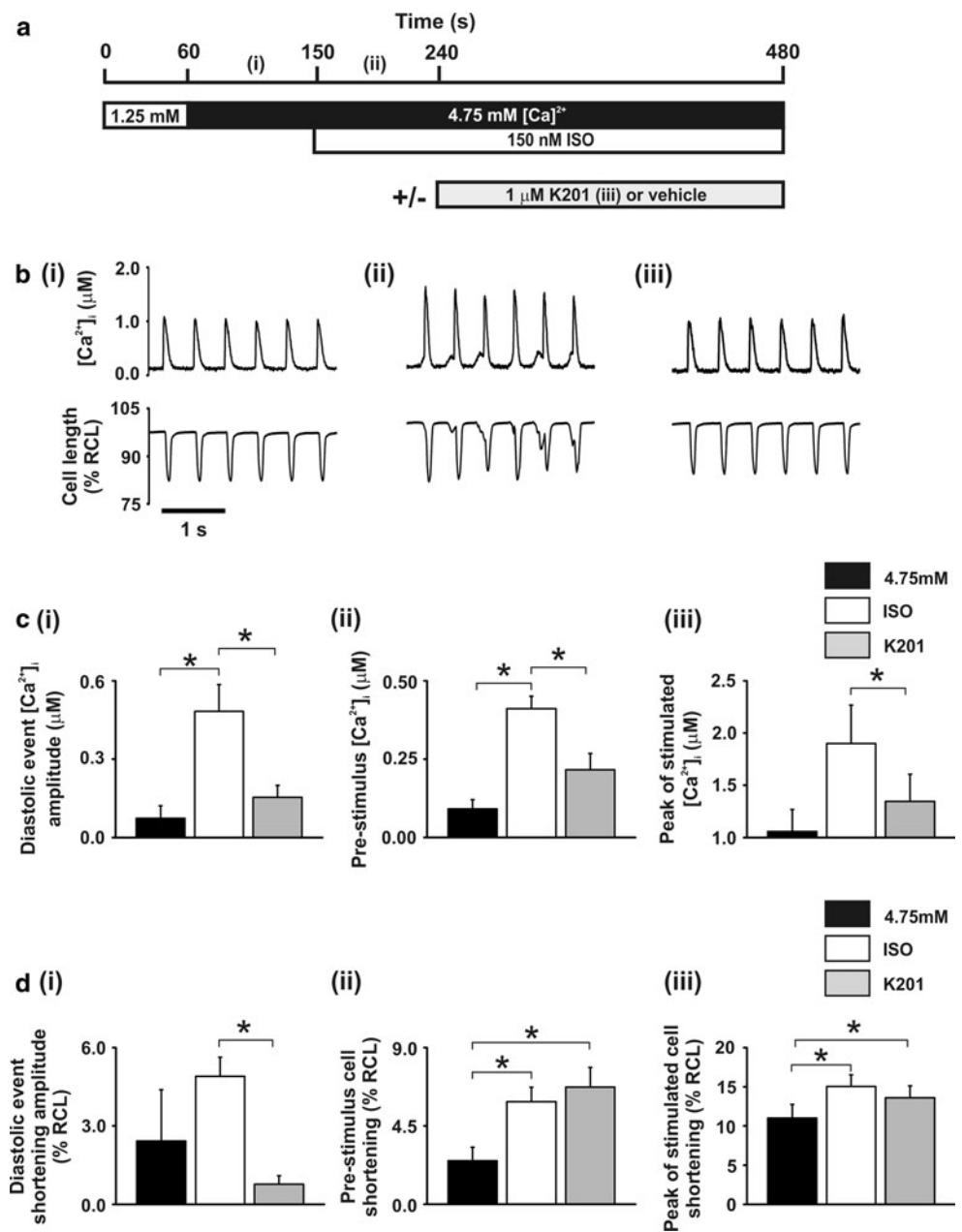
Data were expressed as mean \pm SEM. For Ca^{2+} transient amplitude and Ca^{2+} wave parameters, comparisons were performed by applying the paired Student's *t* test. ANOVA statistics with either a Tukey (Ca^{2+} transient and shortening parameters) or Dunnett (myofilament sensitivity) post-test were used in cases of multiple comparisons. Differences were considered significant when $P < 0.05$.

Results

Measurement of spontaneous diastolic events in isolated ventricular cardiomyocytes

To gain an insight into the cellular mechanisms which contribute to the beneficial effect of K201 in a previously published pharmacologically induced in vivo rat model of diastolic dysfunction [14] isolated rat cells were perfused with a modified KH solution containing the $[\text{Ca}^{2+}]_o$ and additions shown in Fig. 1a. Cells were field stimulated at 2.0 Hz to obtain a sufficient length of time between stimulations in which to characterise diastolic release events during an elevated $[\text{Ca}^{2+}]_o$ and β -adrenergic stimulation. When cells were perfused with 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L isoproterenol (referred to hereafter as “ISO”), the amplitude of diastolic rises of $[\text{Ca}^{2+}]_i$ was markedly

Fig. 1 Effect of 1.0 $\mu\text{mol/L}$ K201 on $[\text{Ca}^{2+}]_i$ and cell shortening **a** perfusion protocol. **b** records of $[\text{Ca}^{2+}]_i$ (upper) and cell length (lower) at points denoted in **a**. **c** mean \pm SEM values of for (i) diastolic event free $[\text{Ca}^{2+}]_i$ amplitude, (ii) pre-stimulus free $[\text{Ca}^{2+}]_i$ and (iii) peak free $[\text{Ca}^{2+}]_i$ of stimulated transient. **d** mean \pm SEM values of cell shortening for (i) diastolic event amplitude, (ii) pre-stimulus and (iii) peak of stimulated transient. *Black bars*, 4.75: 4.75 mmol/L $[\text{Ca}^{2+}]_o$; *White bars*, ISO: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO; *Grey bars*, K201: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO + 1.0 $\mu\text{mol/L}$ K201; $n = 8$, $*P < 0.05$



increased compared to 4.75 mmol/L $[\text{Ca}^{2+}]_o$ alone (referred to hereafter as “4.75”) [73.5 ± 4.8 vs. 483 ± 103 nmol/L; 4.75 vs. ISO: $n = 8$, $P < 0.05$; Fig. 1b(i vs. ii) top panel and c(i)]. These diastolic Ca^{2+} events consistently occurred in parallel with diastolic contractile events (Fig. 1b(i vs. ii) bottom panel and d(i)) and were similar to the diastolic rises of intra-ventricular pressure observed in vivo using a similar protocol [14]. When compared to DMSO vehicle time controls, perfusion with 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L isoproterenol + 1.0 $\mu\text{mol/L}$ K201 for 4 min (referred to hereafter as “K201”) resulted in a cell-to-cell variable response on diastolic Ca^{2+} events. K201 significantly reduced the

magnitude of diastolic Ca^{2+} events in all cells tested (100%) and, in $\sim 50\%$ cells, these were completely abolished (Fig. 1b(iii)). The mean response to K201 was to significantly reduce both the amplitudes of diastolic Ca^{2+} events [483 ± 103 vs. 154 ± 46 nmol/L; ISO vs. K201: $n = 8$, $P < 0.05$; Fig. 1c(i)] and diastolic contractile events [4.90 ± 0.73 – $0.77 \pm 0.33\%$ RCL; ISO vs. K201: $n = 8$, $P < 0.05$; Fig. 1d(i)].

Application of 150 nmol/L ISO resulted in a significant increase in the mean minimum $[\text{Ca}^{2+}]_i$ compared to 4.75 mmol/L $[\text{Ca}^{2+}]_o$ alone (60.9 ± 1.7 vs. 159 ± 33.0 nmol/L; 4.75 vs. ISO: $n = 8$, $P < 0.05$; data not shown). Subsequent perfusion with K201 did not

significantly alter the mean minimum $[Ca^{2+}]_i$ (159 ± 33.0 vs. 120 ± 24.9 nmol/L; ISO vs. K201: $n = 8$, $P > 0.05$; data not shown).

Measurement of the peak stimulated event in isolated ventricular cardiomyocytes

The mean peak of the stimulated $[Ca^{2+}]_i$ transient was significantly reduced by K201 [$1,900 \pm 367$ vs. $1,350 \pm 260$ nmol/L; ISO vs. K201: $n = 8$, $P < 0.05$; Fig. 1c(iii)] as was the mean pre-stimulus $[Ca^{2+}]_i$ [411 ± 40 vs. 216 ± 52 nmol/L; ISO vs. K201: $n = 8$, $P < 0.05$; Fig. 1c(ii)]. In contrast, there was no significant alteration in either the mean peak of the stimulated cell shortening [15.04 ± 1.49 vs. $13.60 \pm 1.52\%$ RCL; ISO vs. K201; $n = 8$, $P > 0.05$; Fig. 1d(iii)], mean pre-stimulus cell shortening [5.89 ± 0.84 vs. $6.74 \pm 1.12\%$ RCL; ISO vs. K201; $n = 8$, $P > 0.05$; Fig. 1d(ii)] or the mean minimum cell shortening (3.23 ± 0.47 vs. $3.43 \pm 0.47\%$ RCL; ISO vs. K201; $n = 8$, $P > 0.05$; data not shown) following application of K201.

Separate voltage clamp measurements on cells under identical conditions (including the presence of an elevated $[Ca^{2+}]_o$) showed only a small decrease in L-type Ca^{2+} channel amplitude in response to $1.0 \mu\text{mol/L}$ K201 ($95.6 \pm 4.4\%$ of timed control level—see Online resource III).

The effect of K201 on the overall electrical activity of ventricular cells was assessed from ECG measurements on isolated Langendorff perfused whole hearts (see Online resource IV). These revealed no significant differences in QT interval at a fixed stimulus rate suggesting no major change in action potential duration in rat hearts exposed to $1.0 \mu\text{mol/L}$ K201.

Effects of K201 on myofilament Ca^{2+} sensitivity

An assessment of the ability of K201 to alter myofilament Ca^{2+} sensitivity was made as this was a previously proposed mechanism to explain the in vivo improvement of function in the rat [14]. β -adrenergic stimulation alters the Ca^{2+} sensitivity of the myofilaments and an elevated Ca^{2+} concentration (i.e. 4.75 mmol/L $[Ca^{2+}]_o$) leads to the production of diastolic contractile events (which can affect the amplitude of the subsequent Ca^{2+} transient) therefore, these experiments were performed in the absence of β -adrenergic stimulation and at lower $[Ca^{2+}]_o$ to ensure that diastolic Ca^{2+} events did not occur. Simultaneous measurements of $[Ca^{2+}]_i$ and cell shortening were used to establish the relationship between Ca^{2+} transient parameters and cell shortening amplitudes in response to varying $[Ca^{2+}]_o$. Cells were field stimulated and perfused with modified KH solutions containing a range of $[Ca^{2+}]_o$ from

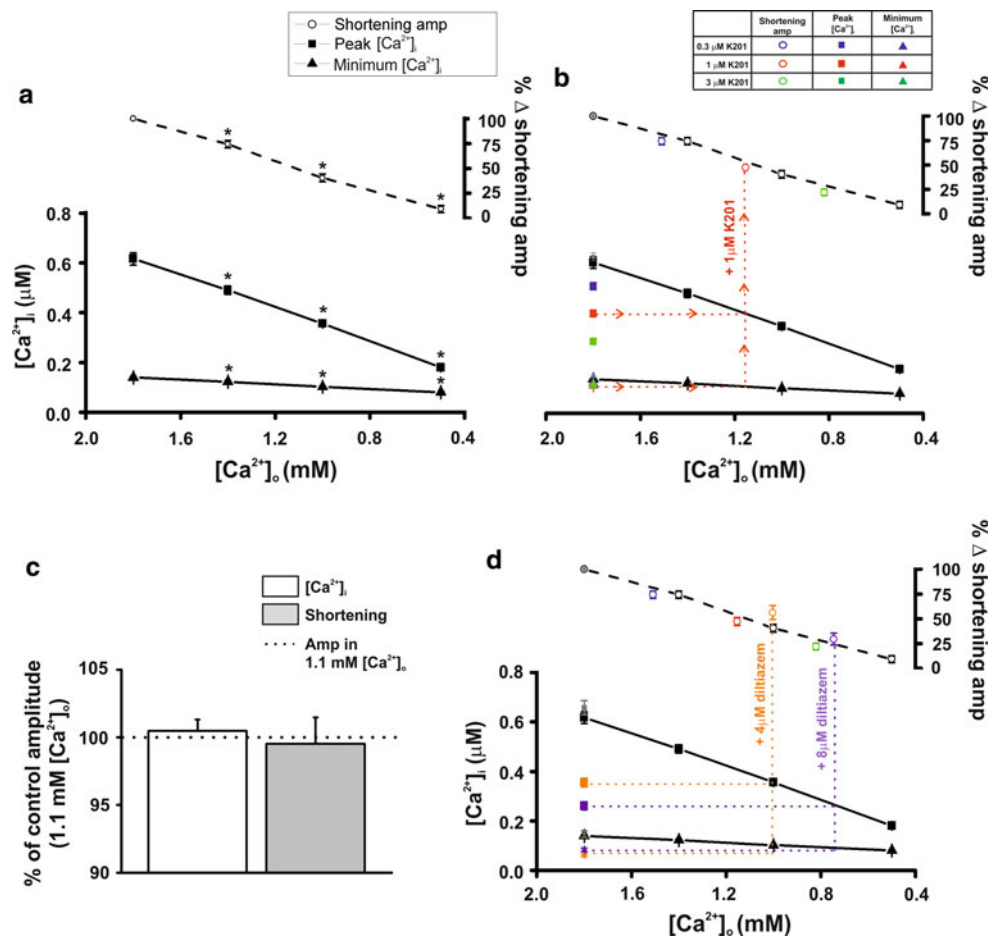
1.8 to 0.5 mmol/L . As shown in Fig. 2a, reducing $[Ca^{2+}]_o$ from 1.8 mmol/L (control) to 0.5 mmol/L resulted in a significant decrease in Ca^{2+} transient peak $[Ca^{2+}]_i$ [617 ± 25 vs. 180 ± 6 nmol/L; 1.8 (control) vs. 0.5 mmol/L $[Ca^{2+}]_o$: $n = 14$, $P < 0.05$] and minimum $[Ca^{2+}]_i$ [140 ± 4 vs. 81 ± 2 nmol/L; 1.8 (control) vs. 0.5 mmol/L $[Ca^{2+}]_o$: $n = 14$, $P < 0.05$]. This was accompanied by an $\sim 90\%$ reduction in cell shortening amplitude [100.0 vs. $9.28 \pm 3.57\%$; 1.8 mmol/L (control) vs. 0.5 mmol/L $[Ca^{2+}]_o$: $n = 14$, $P < 0.05$].

In a second set of experiments, the effect of a range of concentrations of K201 (0.3 – $3.0 \mu\text{mol/L}$) on Ca^{2+} transient parameters and cell shortening amplitudes was examined at a constant $[Ca^{2+}]_o$ (1.8 mmol/L $[Ca^{2+}]_i$). Perfusion with K201 (0.3 – $3 \mu\text{mol/L}$) led to a dose-dependent decrease in Ca^{2+} transient peak and minimum $[Ca^{2+}]_i$ and cell shortening amplitude (Fig. 2b). Addition of $1.0 \mu\text{mol/L}$ K201 (red symbols) significantly reduced Ca^{2+} transient peak $[Ca^{2+}]_i$ [640 ± 16 vs. 409 ± 13 nmol/L; 1.8 mmol/L $[Ca^{2+}]_o$ (control, grey symbols) vs. 1.8 mmol/L $[Ca^{2+}]_o$ + $1.0 \mu\text{mol/L}$ K201: $n = 24$, $P < 0.05$]. Similarly, $1.0 \mu\text{mol/L}$ K201 reduced cell shortening amplitude by $\sim 53\%$ [100.0 vs. $47.25 \pm 3.99\%$; 1.8 mmol/L $[Ca^{2+}]_o$ (control, grey symbol) vs. 1.8 mmol/L $[Ca^{2+}]_o$ + $1.0 \mu\text{mol/L}$ K201: $n = 24$, $P < 0.05$ vs. control].

To determine the effect of K201 on the relationship between Ca^{2+} transient parameters and cell shortening amplitude shown in Fig. 2a, Ca^{2+} transient amplitudes at each K201 concentration were matched to those measured in Ca^{2+} alone by extrapolation of the K201 Ca^{2+} transient peak and minimum points (example for $1.0 \mu\text{mol/L}$ K201 is demonstrated by horizontal red dotted lines, Fig. 2b). Experimentally derived cell shortening amplitudes obtained in 1.8 mmol/L $[Ca^{2+}]_o$ and each concentration of K201 were then plotted at the corresponding $[Ca^{2+}]_o$ value (e.g. for $1.0 \mu\text{mol/L}$ K201, vertical red dotted line). As seen in Fig. 2b, the relationship between Ca^{2+} transient parameters and cell shortening in K201 is the same as that for varying $[Ca^{2+}]_o$ alone. For example, the mean Ca^{2+} transient amplitude in $1.0 \mu\text{mol/L}$ K201 (297 ± 16 nmol/L) predicts a cell shortening amplitude of 49.1% of control which was confirmed by the experimentally measured value of $47.2 \pm 4.0\%$ in $1.0 \mu\text{mol/L}$ K201.

Using the relationship between Ca^{2+} transient parameters and cell shortening amplitude derived when varying $[Ca^{2+}]_o$ alone (Fig. 2a), it was observed that a Ca^{2+} transient amplitude equivalent to that of $1.0 \mu\text{mol/L}$ K201 in 1.8 mmol/L $[Ca^{2+}]_o$ (297 ± 16 nmol/L) was produced by perfusion with $[Ca^{2+}]_o$ of 1.1 mmol/L (without K201). To verify the above negative result of K201 on myofilament Ca^{2+} sensitivity, these two solutions were sequentially perfused onto a separate sub-set of cells to directly assess

Fig. 2 Effect of 1.0 $\mu\text{mol/L}$ K201 on myofilament Ca^{2+} sensitivity **a** relationship between free Ca^{2+} transient parameters (peak and minimum) and cell shortening amplitudes (% shortening) in response to varying external $[\text{Ca}^{2+}]_o$ ($n = 14$, $*P < 0.05$ vs. 1.8 mmol/L for each). **b** as in **a** with additional points obtained in 1.8 mmol/L external $[\text{Ca}^{2+}]_o$ and K201 (0.3, 1.0 and 3.0 $\mu\text{mol/L}$; $n = 24$, $P < 0.05$ for shortening amplitudes at all concentrations vs. 1.8 mmol/L, $P < 0.05$ for peak $[\text{Ca}^{2+}]_i$ at 1 and 3 $\mu\text{mol/L}$ K201 vs. 1.8 mmol/L). **c** mean \pm SEM values for percentage change in amplitude of free $[\text{Ca}^{2+}]_i$ and shortening amplitudes in 1.0 $\mu\text{mol/L}$ K201 expressed relative to control in 1.1 mmol/L external Ca^{2+} (100%; $n = 7$). **d** as in **a** with additional points obtained in 1.8 mmol/L external $[\text{Ca}^{2+}]_o$ and diltiazem (4.0 and 8.0 $\mu\text{mol/L}$ $n = 8$, $P < 0.05$ for shortening amplitudes and peak $[\text{Ca}^{2+}]_i$ at both concentrations vs. 1.8 mmol/L and for minimum $[\text{Ca}^{2+}]_i$ at 8.0 $\mu\text{mol/L}$ diltiazem vs. 1.8 mmol/L)



the effect on cell shortening at equivalent Ca^{2+} transient amplitudes. Figure 2c shows mean data for $[\text{Ca}^{2+}]_i$ and cell shortening obtained in response to 1.0 $\mu\text{mol/L}$ K201 in 1.8 mmol/L Ca^{2+} expressed relative to that at 1.1 mmol/L $[\text{Ca}^{2+}]_o$ alone (100%, without K201; dashed line). The mean data show that at equivalent Ca^{2+} transient amplitudes, K201 does not significantly alter cell shortening (transient amplitude: $100.48 \pm 0.85\%$ of control; shortening amplitude: $99.53 \pm 1.95\%$ of control; $n = 7$, $P > 0.05$ for each).

To expand this finding, a third set of experiments were performed utilising diltiazem as a tool to alter Ca^{2+} transient parameters to a similar degree as reducing $[\text{Ca}^{2+}]_o$ alone. Diltiazem (4.0 and 8.0 $\mu\text{mol/L}$) caused a dose-dependent decrease in transient peak and minimum $[\text{Ca}^{2+}]_i$ (Fig. 2d). Equivalent Ca^{2+} transient amplitudes in diltiazem were matched to the relationship derived in Ca^{2+} alone (in Fig. 2a) and cell shortening amplitudes were plotted as before. At equivalent Ca^{2+} transient amplitudes, cell shortening in the presence of diltiazem was not significantly different from the levels predicted by altering $[\text{Ca}^{2+}]_o$ alone.

Effects of K201 on SR function

Following 1.5 min of 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO then 4.0 min perfusion with or without 1.0 $\mu\text{mol/L}$ K201, rapid application of 10.0 mmol/L caffeine was used to assess SR Ca^{2+} content (Fig. 3a(i)). Perfusion with K201 resulted in no significant alteration of SR free Ca^{2+} content as determined by the mean amplitude of the caffeine-induced Ca^{2+} transient [$1,310 \pm 230$ vs. $1,400 \pm 260$ nmol/L; 4.75 mmol/L ISO vs. K201; $n = 8$, $P > 0.05$; Fig. 3a(ii)]. The rate of decline of the caffeine-induced transients is a measure of the rate of sarcolemmal Ca^{2+} extrusion, these rates were not altered following K201 (1.35 ± 0.15 vs. 1.21 ± 0.15 $\mu\text{mol/L s}^{-1}$; ISO vs. K201; $n = 8$, $P > 0.05$; data not shown).

Given the dramatic reduction in the amplitude of diastolic Ca^{2+} release events by K201, the $[\text{Ca}^{2+}]_i$ immediately preceding the stimulus (pre-stim) was lower after K201 (see Fig. 1c(ii)). The consequences of this alteration in diastolic Ca^{2+} level at the point of action potential-induced Ca^{2+} release must be considered when assessing the effect of K201 on the Ca^{2+} transient amplitude. To

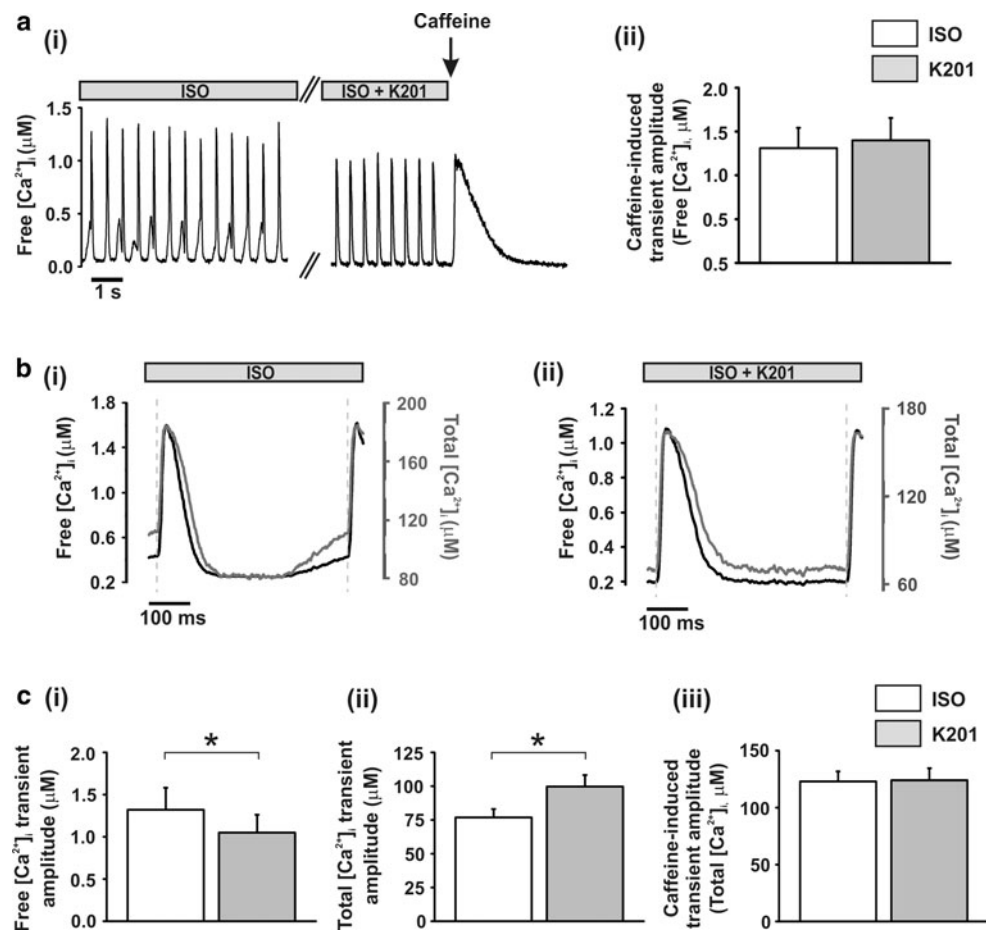


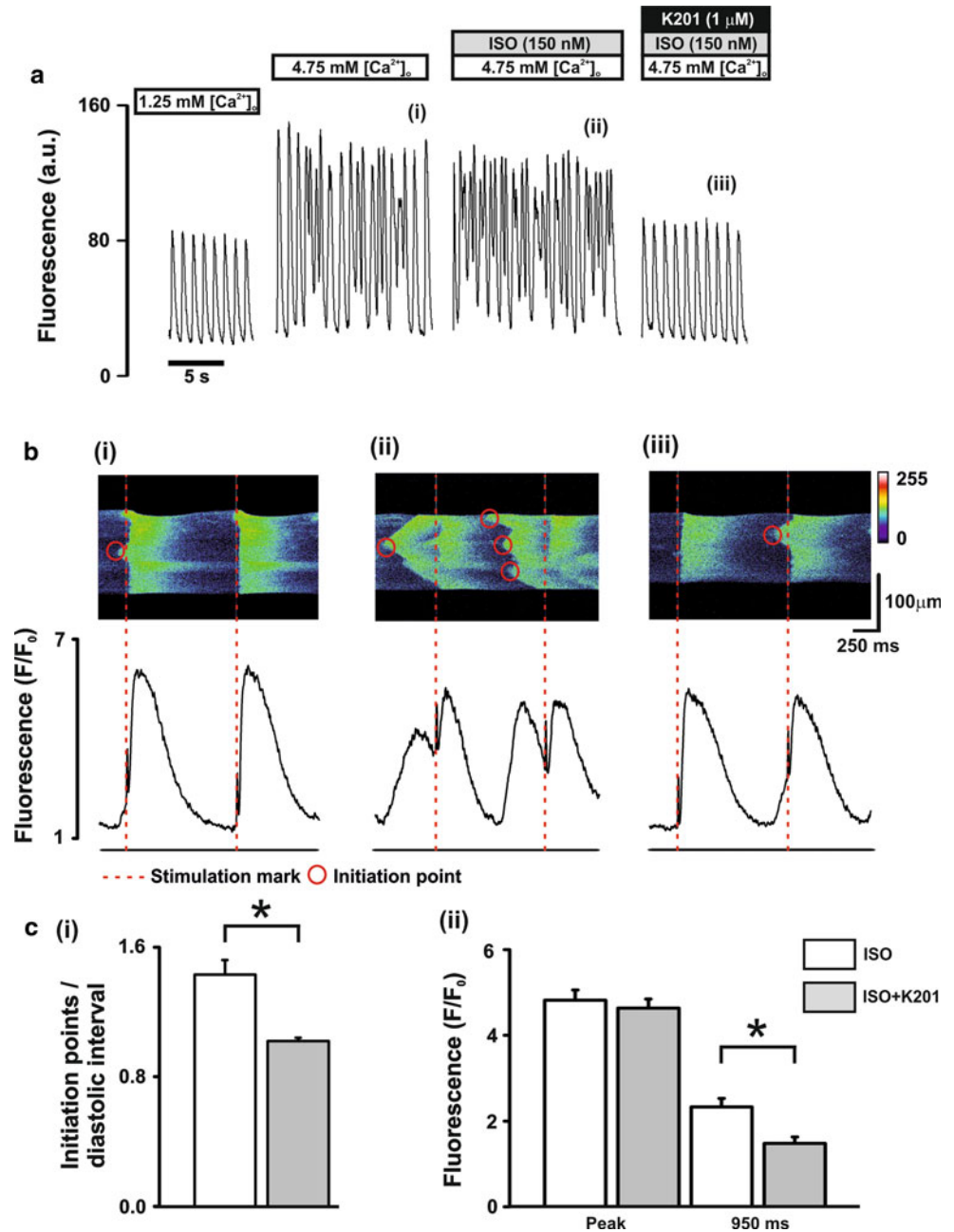
Fig. 3 Effect of 1.0 $\mu\text{mol/L}$ K201 on Ca^{2+} transient amplitude and SR Ca^{2+} content **a** (i) typical records of free $[\text{Ca}^{2+}]_i$ in a cell perfused with 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO then 1 $\mu\text{mol/L}$ K201 with subsequent caffeine application (arrow); (ii) mean \pm S.E.M. values of caffeine-induced free Ca^{2+} transient amplitude ($n = 8$). **b** Average transient in free Ca^{2+} (black trace) and total Ca^{2+} (grey trace) in the presence of (i) ISO: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO and (ii) ISO + K201: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L

ISO + 1.0 $\mu\text{mol/L}$ K201; dashed lines denote stimulus mark. **c** mean \pm SEM values of (i) free Ca^{2+} transient amplitude ($n = 11$) and (ii) total Ca^{2+} transient amplitude ($n = 11$) both measured from the peak Ca^{2+} minus pre-stim Ca^{2+} of their respective averaged traces; (iii) caffeine-induced total Ca^{2+} transient amplitude. ($n = 8$). White bars, ISO: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO, Grey bars, K201: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO + 1.0 $\mu\text{mol/L}$ K201; * $P < 0.05$

allow for varying degrees of intracellular buffering of SR Ca^{2+} release under these two conditions, mean traces were converted to total cellular $[\text{Ca}^{2+}]$ using previously reported intracellular buffer concentrations and affinities (Fig. 3b(i, ii), [8]). The Ca^{2+} transient amplitude was measured by subtracting the pre-stim from the peak of the stimulated transient for the average signals converted to free $[\text{Ca}^{2+}]$ and total $[\text{Ca}^{2+}]$. This analysis revealed that whilst K201 produced a significant reduction in the amplitude of the free Ca^{2+} transient [1.32 ± 0.26 vs. 1.05 ± 0.21 $\mu\text{mol/L}$; ISO vs. K201: $n = 11$, $P < 0.05$; Fig. 3c(i)] there was a significant increase in the amplitude of the total Ca^{2+} transient [76.8 ± 6.3 vs. 99.8 ± 8.5 $\mu\text{mol/L}$; ISO vs. K201: $n = 11$, $P < 0.05$; Fig. 3c(ii)]. Conversion of the caffeine-induced Ca^{2+} transient to total $[\text{Ca}^{2+}]_i$ showed no significant difference between SR content in ISO and K201 (Fig. 3c(iii)).

Characterisation of the stimulated Ca^{2+} transients shown in Fig. 1 revealed that whilst 1.0 $\mu\text{mol/L}$ K201 did not alter the maximum rate of rise of the stimulated Ca^{2+} transient it significantly decreased both the maximum rate of fall [$4.3 \times 10^{-5} \pm 1.1 \times 10^{-5}$ vs. $2.4 \times 10^{-5} \pm 6.4 \times 10^{-6}$ M s^{-1} ; ISO vs. K201: $n = 8$, $P < 0.05$; Online resource V(a)] and the rate of fall at 500 nmol/L $[\text{Ca}^{2+}]_i$ [$1.8 \times 10^{-5} \pm 2.2 \times 10^{-6}$ vs. $1.1 \times 10^{-5} \pm 1.3 \times 10^{-6}$ M s^{-1} ; ISO vs. K201: $n = 8$, $P < 0.05$; Online resource V (b and c), respectively]. Separate direct measurements of the effect of 1.0 $\mu\text{mol/L}$ K201 on SERCA activity showed no significant inhibition of the rate constant of decay of free $[\text{Ca}^{2+}]_i$ [index of SERCA function in a permeabilised cell assay as described in Online resource Materials, Online resource V(d)]. While the effect of K201 was not apparent at 1.0 $\mu\text{mol/L}$, at the higher concentration

Fig. 4 Characterisation of diastolic Ca^{2+} release events **a** perfusion protocol (*upper*) and typical records of Fluo-3 fluorescence (*lower*). **b** Typical line scan images (*upper*) and corresponding fluorescence profiles (*lower*) in the presence of (i) 4.75 mmol/L $[\text{Ca}^{2+}]_o$, (ii) 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO and (iii) 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO + 1.0 $\mu\text{mol/L}$ K201; *dashed lines (red)* represent stimulus mark, *circles* represent wave initiation points. **c** mean \pm SEM values of (i) number of wave initiation points/diastolic interval and (ii) fluorescence signals measured at the peak and at 950 ms post stimulation. *White bars*, ISO: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO; *Grey bars*, ISO + K201: 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO + 1.0 $\mu\text{mol/L}$ K201; $n = 11$, $*P < 0.05$



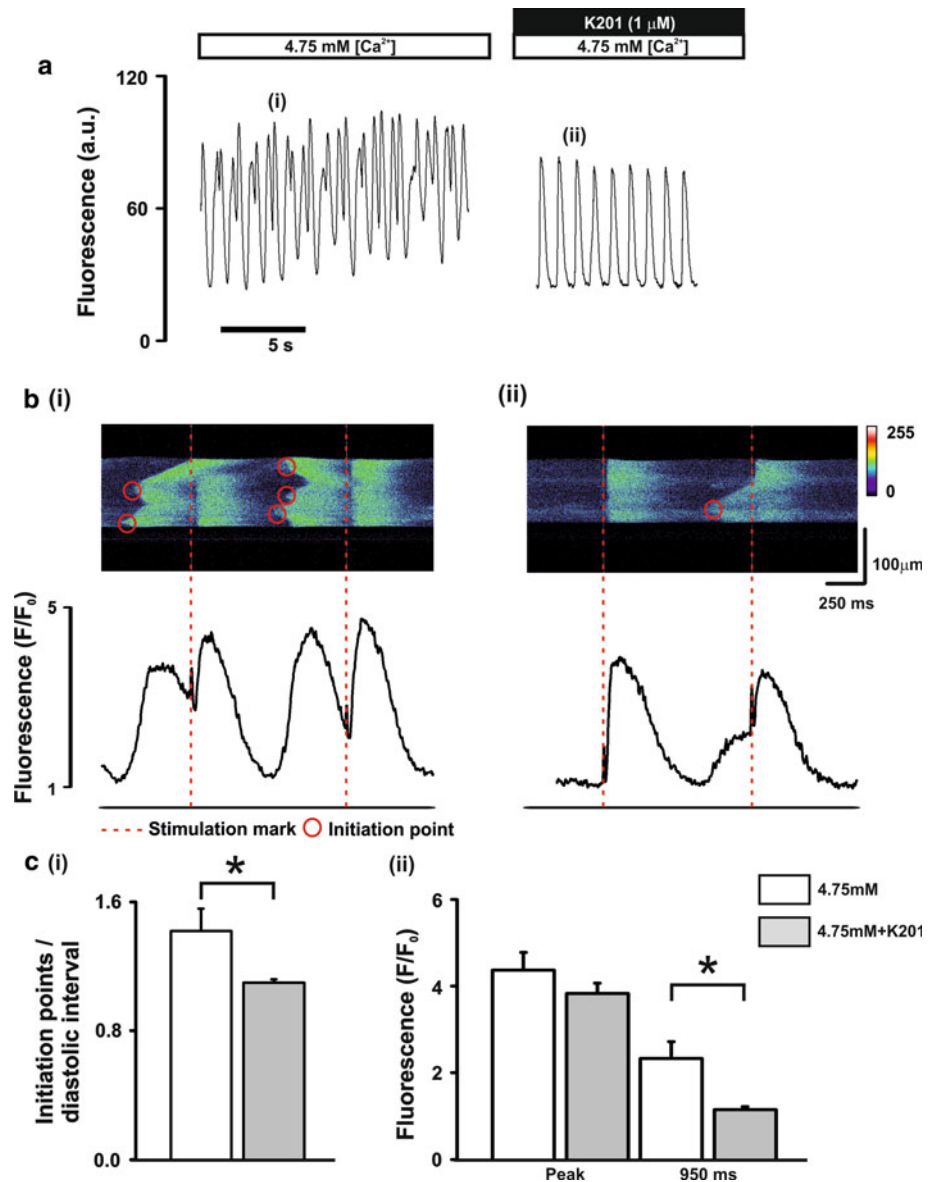
of 10.0 $\mu\text{mol/L}$, the rate constant of decay was significantly reduced to $73.9 \pm 2.5\%$ [Online resource V(e), $n = 4$, $P < 0.05$].

Confocal measurements of diastolic Ca^{2+} events during β -adrenergic stimulation

To visualise the effect of K201 on individual diastolic Ca^{2+} release events at an enhanced spatiotemporal resolution, a set of experiments was performed using confocal imaging of rat cardiomyocytes perfused with the same $[\text{Ca}^{2+}]_o$ and additions as in Fig. 1 (Fig. 4a). Line scan confocal imaging revealed a synchronous rise of $[\text{Ca}^{2+}]_i$ along the length of

the scan line at each stimulation point (Fig. 4b; red dashed lines denote stimulation mark). Upon perfusion with 4.75 mmol/L $[\text{Ca}^{2+}]_o$ + 150 nmol/L ISO, spontaneous diastolic Ca^{2+} events were visualised in the line scan (Fig. 4b(ii) vs. b(i) top panels) and corresponding fluorescence intensity profiles (Fig. 4b(ii) vs. b(i) bottom panels). Diastolic Ca^{2+} events were initiated at discrete points along the scan line (Fig. 4b(ii) top panel) and then propagated along the cell length identifying these diastolic events as being the result of multiple Ca^{2+} waves. Initiation points of the Ca^{2+} waves were identified on the basis of evidence from the line scan image of propagation away from a separate site. The number of initiation points which

Fig. 5 Characterisation of diastolic Ca^{2+} release events in the absence of ISO **a** perfusion protocol (*upper*) and typical records of Fuo-3 fluorescence (*lower*). **b** typical line scan images (*upper*) and corresponding fluorescence profiles (*lower*) in the presence of (i) 4.75 mmol/L $[\text{Ca}^{2+}]_o$ and (ii) 4.75 mmol/L $[\text{Ca}^{2+}]_o + 1.0 \mu\text{mol/L}$ K201; *dashed lines (red)* represent stimulus mark, *circles* represent wave initiation points. **c** mean \pm SEM values of (i) number of wave initiation points/diastolic interval and (ii) fluorescence signals measured at the peak and at 950 ms post stimulation. *White bars*, 4.75: 4.75 mmol/L $[\text{Ca}^{2+}]_o$; *Grey bars*, 4.75 + K201: 4.75 mmol/L $[\text{Ca}^{2+}]_o + 1.0 \mu\text{mol/L}$ K201; $n = 4$, $*P < 0.05$



led to Ca^{2+} waves was markedly increased when ISO was added to the perfusing solution and typically resulted in multiple waves in the diastolic interval (Fig. 4b(ii) top panel). Application of 1.0 $\mu\text{mol/L}$ K201 caused a significant reduction in the frequency of diastolic events (12.45 ± 1.36 vs. 7.55 ± 1.53 diastolic events/15 s period; ISO vs. K201: $n = 11$, $P < 0.05$ —data not shown) and, as illustrated in Fig. 4b(iii) and c(i), a significant reduction in the initiation points/diastolic interval in the remaining diastolic Ca^{2+} release events [1.43 ± 0.09 vs. 1.02 ± 0.02 ; ISO vs. K201: $n = 11$, $P < 0.05$; Fig. 4c(i)]. This was accompanied by no significant change in stimulated transient peak fluorescence but a significant reduction in the fluorescence measured at 950 ms post stimulation compared to control [2.33 ± 0.20 vs. 1.48 ± 0.15 F/F_0 ; ISO vs. K201: $n = 11$, $P < 0.05$; Fig. 4c(ii)]. Measurements of Ca^{2+} spark events during the

diastolic period revealed a significant decrease in Ca^{2+} spark amplitude (3.24 ± 0.18 vs. 2.84 ± 0.16 ; ISO vs. K201: $n = 416$ control sparks and $n = 183$ K201 sparks, $P < 0.05$) and a trend towards a decrease in Ca^{2+} spark frequency (see Online resource VI).

Confocal measurements of diastolic Ca^{2+} events during elevated $[\text{Ca}^{2+}]_o$.

A sub-set of cells exhibited a propensity to produce diastolic Ca^{2+} events when exposed to 4.75 mmol/L $[\text{Ca}^{2+}]_o$ without ISO (Fig. 5a). Confocal images obtained from these cells showed a synchronous rise of $[\text{Ca}^{2+}]_i$ at each stimulation point and multiple waves originating at various initiation points during the diastolic interval (Fig. 5b(i)). Application of 1.0 $\mu\text{mol/L}$ K201 without ISO (referred to

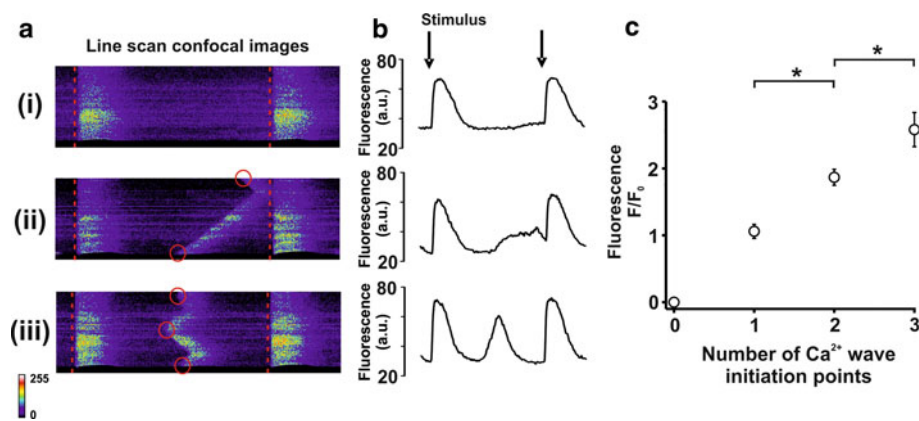


Fig. 6 Characterisation of Ca^{2+} wave initiation points **a** (i–iii) typical line scan images with varying numbers of initiation points; *red lines* and *circles* denote stimulus mark and initiation points, respectively. **b** (top to bottom): fluorescence intensity profiles corresponding to the images in **a**; *arrows* represent the time of stimulation. **c** mean \pm SEM

hereafter as 4.75 + K201) markedly reduced the frequency of these initiation points/diastolic interval [1.33 ± 0.13 vs. 1.03 ± 0.02 ; 4.75 vs. 4.75 + K201: $n = 4$, $P < 0.05$; Fig. 5b(ii), c(i)] along with a significant decrease in the diastolic fluorescence measured at 950 ms post stimulation compared to 4.75 mmol/L $[\text{Ca}^{2+}]_o$ without K201 [2.33 ± 0.39 vs. $1.15 \pm 0.07 F/F_0$; 4.75 vs. 4.75 + K201: $n = 4$, $P < 0.05$; Fig. 5c(ii)]. No significant change in stimulated transient peak fluorescence was observed.

Confocal measurements of Ca^{2+} wave initiation points and diastolic Ca^{2+} event amplitude

To examine the relationship between the number of initiation points per diastolic event and corresponding diastolic event amplitude, the confocal images of a number of cells ($n = 8$) perfused with 4.75 mmol/L $[\text{Ca}^{2+}]_o$ and ISO were examined as in Fig. 6a(i–iii) with respective fluorescence intensity profiles (Fig. 6b top–bottom). Number of initiation points (highlighted by red circles—Fig. 6a) per diastolic event was plotted against the amplitude of that event (Fig. 6c). The mean data showed a positive correlation between the two parameters with significant differences between 1, 2 and 3 initiation points (1.06 ± 0.1 , 1.87 ± 0.1 , $2.58 \pm 0.26 F/F_0$; $P < 0.05$; Fig. 6c), respectively.

Discussion

We have provided for the first time a detailed characterisation of the effects of K201 on the stimulated Ca^{2+} transient, spontaneous diastolic SR mediated Ca^{2+} release and contractile events during application of β -adrenergic stimulation and an elevated $[\text{Ca}^{2+}]_o$ in rat cardiomyocytes. Our main findings are: (a) K201 has no effect on stimulated

data showing the relationship between diastolic Ca^{2+} event initiation points and diastolic Ca^{2+} event amplitude taken from 8 cells in Fig. 4 (1, 2 and 3 initiation points, $n = 42$, 30 and 6 diastolic Ca^{2+} events, respectively, $*P < 0.05$)

cell shortening but significantly reduces spontaneous diastolic contractile events induced by elevated Ca^{2+} loading, (b) K201 produces a small but significant reduction in stimulated free Ca^{2+} transient amplitude and significantly reduces spontaneous Ca^{2+} release events with no alteration in SR Ca^{2+} content, (c) when intracellular buffering is taken into account, K201 produces a significant increase in total Ca^{2+} transient amplitude, (d) K201 had no effect on myofilament Ca^{2+} sensitivity thereby discounting this previously proposed mechanism to explain the in vivo improvement of function in the rat [14], (e) 1.0 $\mu\text{mol/L}$ K201 does not inhibit SERCA in rat cardiomyocytes (which is in contrast to the effect in rabbit [18]) and (f) spontaneous diastolic Ca^{2+} events occurred in the form of multiple propagating Ca^{2+} waves (each with an initiating point) and that K201 acts to reduce both the event frequency and number of initiation points per diastolic event.

Comparison between the effect of K201 on diastolic contractions in vivo and in isolated cardiomyocytes

A previous study has demonstrated that in vivo application of a β -adrenergic agonist and an elevated $[\text{Ca}^{2+}]_o$ led to the production of diastolic contractions resulting in a raised intra-ventricular pressure measured at the R wave [14]. This pharmacological intervention resulted in a model of severe acute diastolic dysfunction without a significant change in systolic peak pressure. Application of 1.0 $\mu\text{mol/L}$ K201 in vivo reduced the occurrence of the diastolic contractures and significantly reduced the mean intra-ventricular pressure measured at the R wave with no significant alteration of the peak pressure. With the caveat that detailed comparison of contractile function between isolated cardiomyocyte and whole heart experiments is

problematic, the aforementioned effect of K201 on the mechanical function of the whole rat heart demonstrates some similarities to shortening measurements in isolated rat cardiomyocytes performed in this study (Fig. 1). In the present study, β -adrenergic stimulation of ventricular cardiomyocytes perfused with an elevated $[Ca^{2+}]_o$ also led to the production of spontaneous diastolic cellular contractions. The amplitude of the spontaneous diastolic contractions was significantly reduced by K201 (to $\sim 16\%$ of control) with no alteration of the stimulated shortening amplitude. One difference between the *in vivo* rat study and the isolated cardiomyocyte experiments is the response of the cells in the current study with elevated $[Ca^{2+}]_o$ to ISO, which resulted in a positive inotropy (Fig. 1d(iii)). A trend to positive inotropy also existed within the *in vivo* model (reflected by the increase in left ventricular pressure) after 25 min of an elevated $[Ca^{2+}]_o$ and β -adrenergic stimulation but this did not reach significance. Reasons for this discrepancy include the fact that the single cardiomyocytes were (a) only exposed to β -adrenergic stimulation and an elevated $[Ca^{2+}]_i$ for ~ 5.0 min and (b) field stimulated (paced) and therefore not affected by the changes in heart rate which accompany an *in vivo* response.

The relationship between the stimulated Ca^{2+} transient and cell shortening amplitude

The current data reveal for the first time the relationship between the effect of K201 on stimulated Ca^{2+} transient amplitude together with the degree of cell shortening. The results demonstrate that although $1.0 \mu\text{mol/L}$ K201 significantly reduced the stimulated free Ca^{2+} transient amplitude by 16.1% [$P < 0.05$; Fig. 3a(ii)], the stimulated shortening amplitude was not significantly affected. No data exist in the literature as to the effect of K201 on the myofilament sensitivity to $[Ca^{2+}]_i$. To investigate this, the relationship between Ca^{2+} transient amplitude and degree of cell shortening was determined by lowering $[Ca^{2+}]_o$ to various levels (Fig. 2). This relationship was unaltered in the presence of a range of K201 concentrations or an alternative benzothiazepine derivative (diltiazem) thereby demonstrating that K201 does not alter the sensitivity of the myofilaments under these conditions. Further verification of this was obtained via direct myofilament measurements performed in permeabilised rat ventricular cardiomyocytes at a free $[Ca^{2+}]_i$ of 840 nmol/L (Online resource II) and confirmed at 375 nmol/L (data not shown). The latter concentration is equivalent to the mean $[Ca^{2+}]_i$ observed during perfusion of $1.0 \mu\text{mol/L}$ K201 during β -adrenergic stimulation and an elevated $[Ca^{2+}]_o$ (359 ± 43.9 ; $n = 8$; Fig. 1). Thus, the absence of a negative inotropic effect of K201 may be based on the

non-linear relationship between peak $[Ca^{2+}]_i$ and shortening under conditions of Ca^{2+} overload. It is not clear why K201 did not alter mean minimum $[Ca^{2+}]_i$; this may reflect the lack of effect of K201 on sarcolemmal extrusion as previously reported [18].

The effect of K201 on the SR Ca^{2+} content

Previous studies have attributed the beneficial effect of K201 on diastolic cardiac function to the ability of K201 to ensure maximal binding of FKBP12.6 to the RyR2 thus reducing the open probability of RyR2 [26]. The resultant effect would be to prevent diastolic Ca^{2+} release and subsequently to reduce arrhythmias and diastolic contractions. Venetucci et al. [25] have demonstrated in rat cardiomyocytes that the sole inhibitory effect on RyR2 by tetracaine (a drug which decreases the open probability of RyR2) can lead to inhibition of diastolic Ca^{2+} events between depolarisations but leads to an increase in the amplitude of systolic Ca^{2+} transient and SR Ca^{2+} content [4, 25]. The current study observed significant inhibition of diastolic Ca^{2+} spark activity by $1.0 \mu\text{mol/L}$ K201 supporting the concept that K201 reduces the activity of RyR2 but no significant difference in SR content was observed. However, due to the cell-to-cell variability in the caffeine response it is acknowledged that the measurements in the current study may have been unable to discriminate a ~ 10 – 12% change in SR Ca^{2+} content. Despite this limitation, it should be recognised that K201 is not the only drug which demonstrates inhibition of RyR2 without a concomitant increase in SR Ca^{2+} content. Flecainide has been reported to reduce Ca^{2+} wave amplitude in permeabilised rat cardiomyocytes without alteration in the SR Ca^{2+} content or SERCA activity. This is thought to have been achieved by inhibition of RyR2 via an open state block; under these circumstances comparable leak is achieved via significantly higher spark rates despite a significantly reduced “spark mass” [7].

The effect of K201 on the Ca^{2+} transient amplitude

K201 has been shown to prevent diastolic dysfunction and improve systolic function during Ca^{2+} overload conditions in terminally failing human myocardial preparations [24]. In contrast to the current study, no direct measurements of the Ca^{2+} transient or $[Ca^{2+}]_i$ were performed. In another previous study by the authors, on this occasion in rabbit cardiomyocytes, the amplitude of depolarisation-induced Ca^{2+} transients was reduced by $1.0 \mu\text{mol/L}$ K201 but this did not reach significance [18]. Cellular shortening measurements were not reported in this previous work. The current study permits further insight into the effects of K201 using a fluorophore (Fura 4F AM) with a lower

affinity which is able to determine the systolic $[Ca^{2+}]_i$ transient with more accuracy [28]. These novel data suggest that the action potential-induced free Ca^{2+} transients demonstrate a small but significant reduction in amplitude in response to 1.0 $\mu\text{mol/L}$ K201. These changes occurred in the absence of major changes in ventricular electrophysiology (ECG recordings; Online resource IV) or the activity of common Ca^{2+} handling proteins, e.g. SERCA or NCX (see Online Resource V). In particular, 1.0 $\mu\text{mol/L}$ K201 caused only a small decrease ($\sim 5\%$) in I_{Ca} amplitude in the presence of an elevated $[Ca^{2+}]_o$. This contrasts with a previous study which showed a reduction of I_{Ca} peak by 22% in rat cardiomyocytes [10], but is similar to the $\sim 3\%$ reduction observed in rabbit cardiomyocytes [18]. This slight reduction in I_{Ca} ($\sim 5\%$) would not be expected to cause a significant ($>5\%$) change in Ca^{2+} transient amplitude [9]. Superficially, it would appear difficult to reconcile the absence of the effects of K201 on SR Ca^{2+} content and I_{Ca} with the reduction in the peak of the stimulated free Ca^{2+} transient. Particularly, since K201 was very effective in inhibiting spontaneous diastolic Ca^{2+} release which should result in more Ca^{2+} being available for release on the subsequent stimulus. The reconciliation of these data comes when allowance is made for the intracellular buffering which, as shown by previous studies [8], is highly nonlinear over the range of $[Ca^{2+}]_i$ measured in this study ($\sim 75\text{--}2,000$ nmol/L). Using the values of reported intracellular buffer concentrations and affinities, the free $[Ca^{2+}]_i$ in this study were converted to total cellular $[Ca^{2+}]_i$. On this basis, the amplitude of the Ca^{2+} transient (peak-pre-stim) when expressed as total $[Ca^{2+}]_i$ indicates that on addition of K201, the amount of Ca^{2+} release from the SR increased (to $\sim 140\%$ of control). This was not observed in terms of free $[Ca^{2+}]_i$ because although more SR Ca^{2+} appeared to be released after K201, the cellular buffer value was higher resulting in a decreased peak systolic Ca^{2+} level. Increased Ca^{2+} released from the SR on abolition of spontaneous Ca^{2+} release is consistent with studies by Venetucci et al. [25] who also reported increased SR Ca^{2+} content on abolition of Ca^{2+} waves. This was not evident from this study, but the variability of the data would prevent increases of SR content of $\sim 10\%$ being detected; an increase in SR Ca^{2+} content as small as 10% would be sufficient to explain an increase of SR Ca^{2+} release of $\sim 140\%$ [23].

Other contributing factors which were not investigated include alteration in the intracellular $[Na^+]_i$. Future work using the voltage clamp technique and monitoring of intracellular $[Na^+]_i$ may uncover to what degree K201 is able to alter the balance between Ca^{2+} influx and efflux and control for certain parameters which are not achievable with the field stimulation technique.

Confocal imaging reveals characteristics of diastolic Ca^{2+} events

SR mediated Ca^{2+} release occurs via clusters of ryanodine receptors. During diastole this occurs in the form of either localised (Ca^{2+} sparks) or propagating whole cell Ca^{2+} release (Ca^{2+} waves). Ca^{2+} waves are recognised as events which could potentially lead to delayed after depolarisations (DADs) and triggered arrhythmias. A DAD occurs when a transient inward current is produced upon extrusion of SR-mediated diastolic Ca^{2+} via NCX. The ability of this inward current to generate a DAD is, amongst other factors, dependent upon the size of the Ca^{2+} wave and the inward current generated [19]. While there is an established link between Ca^{2+} waves and electrical abnormalities, the link between Ca^{2+} waves and mechanical dysfunction is less clear. To ascertain the spatiotemporal properties of the spontaneous diastolic Ca^{2+} release events, confocal microscopy of ventricular cardiomyocytes was employed using a similar protocol to that in Fig. 1. The experiments revealed that the diastolic events were predominantly in the form of propagating Ca^{2+} waves (Figs. 4, 5), which were significantly reduced in frequency and amplitude by 1.0 $\mu\text{mol/L}$ K201. Characterisation of these events revealed multiple initiating points, the number of which was reduced upon application of 1.0 $\mu\text{mol/L}$ K201. Thus, whilst K201 acts to significantly reduce the probability of spontaneous diastolic events (by $\sim 50\%$) it also acts to modulate the characteristics of the remaining events. The significance of the transition in the latter is illustrated in Fig. 6. Diastolic Ca^{2+} events with multiple initiation points (Fig. 6a(iii vs. ii)) tend towards being more synchronous in activation (similar to the stimulated Ca^{2+} transient (Fig. 6a(i)). Analysis of the whole cell fluorescence (Fig. 6b) reveals that the larger number of initiation points results in a larger amplitude of spontaneously released Ca^{2+} from the SR (Fig. 6b (bottom)) and hence may increase the propensity to generate a transient inward current. This is summarised in the mean data presented in Fig. 6c, which demonstrate that for every extra initiation point per diastolic Ca^{2+} event during a short diastolic interval, the amplitude of the global Ca^{2+} release increases by ~ 1.8 times. Conversely, a reduction in the number of these initiation points (Fig. 6b (middle)) would be expected to lead to a reduction in Ca^{2+} available for extrusion at any one time by NCX. An insight into the net effect of this response to K201 in a multi-cellular preparation can be seen in the averaged traces of $[Ca^{2+}]_i$ and contraction (Online Resource Ia).

Synchronicity of initiation points in the ISO data set (Fig. 4) was measured using the time between the first and last initiation point in each diastolic interval. This value was then correlated with the Ca^{2+} spark frequency in that cell. In cells with low spark frequency ($6.0 \pm 1.1/100$ $\mu\text{m/s}$), the

initiation points were on average 120.4 ± 13.9 ms between the first and last wave in a diastolic interval. In cells with a much higher spark frequency ($35.4 \pm 1.1/100 \mu\text{m/s}$), the initiation points were on average 69.4 ± 7.3 ms between the first and last wave in a diastolic interval. This proved to be a significant correlation ($P < 0.05$). In these two sub-groups, Ca^{2+} spark amplitude was, however, not significantly different. Therefore, while the action of K201 to reduce Ca^{2+} spark frequency (albeit not significantly, see online resource VI) may lead to a reduction in the synchronicity of Ca^{2+} initiation points, the effect is not enough to explain the reduction in number of Ca^{2+} initiation points.

Is the effect of K201 on Ca^{2+} waves related to β -adrenergic antagonism?

It is possible that the effect of K201 to reduce diastolic Ca^{2+} release event amplitude and number of initiation points in this study is due to an ability of K201 to antagonise the effect of β -adrenergic stimulation. While the results in Fig. 5 cannot discount this hypothesis, it seems unlikely given that in the sub-set of cells where Ca^{2+} waves were produced in the absence of β -adrenergic stimulation, the effect of K201 to limit these Ca^{2+} waves was identical to that in the presence of β -adrenergic stimulation (Fig. 4).

Summary and conclusion

The anti-arrhythmic effect of K201 has been attributed to reducing the open probability of RyR2 thus preventing excessive diastolic SR mediated Ca^{2+} leak [26]. This study provides several new mechanistic insights which advance our knowledge of K201. For the first time, we have examined the effect of K201 on intracellular Ca^{2+} and contractile function of isolated rat cardiomyocytes and have shown the drug significantly inhibits diastolic Ca^{2+} release and their associated contractile events leading to an increased action potential-induced SR Ca^{2+} release. These effects occur in the absence of altered myofilament Ca^{2+} sensitivity. Our data also provide greater insight into the effect of K201 on the spatiotemporal properties of Ca^{2+} handling introducing the concept that a reduction in Ca^{2+} wave initiation points has the potential to be anti-arrhythmic due to the limitation of the amplitude of the Ca^{2+} wave and subsequent increase in transient inward current. This may therefore be a route through which both the deleterious arrhythmogenic and mechanical effects of Ca^{2+} waves can be limited.

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Conflict of interest NK is employed in the position of Chairman by Aetas Pharma Co. Ltd (the company supplying K201). Aetas Pharma Co. Ltd. agree with this submission to Basic Research in Cardiology.

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