Systolic $[Ca^{2+}]_i$ regulates diastolic levels in rat ventricular myocytes.

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Key points summary

- For the heart to function as a pump, intracellular calcium concentration ($[Ca^{2+}]_i$) must increase during systole to activate contraction and then fall, during diastole, to allow the myofilaments to relax and the heart to refill with blood.
- This study investigates the control of diastolic $[Ca^{2+}]_i$ in rat ventricular myocytes.
- We now show that diastolic $[Ca^{2+}]_i$ is increased by manoeuvres which decrease sarcoplasmic reticulum function. This is accompanied by a decrease of systolic $[Ca^{2+}]_i$ such that the time-averaged $[Ca^{2+}]_i$ remains constant.
- Here we report that diastolic $[Ca^{2+}]_i$ is controlled by the balance between Ca^{2+} entry and Ca^{2+} efflux during systole.
- Our results identify a novel mechanism whereby changes of the amplitude of the systolic Ca transient control diastolic $[Ca^{2+}]_i$.

Abstract

$[Ca^{2+}]_i$ must be low enough in diastole so that the ventricle is relaxed and can refill with blood. Interference with this will impair relaxation. The factors responsible for regulation of diastolic $[Ca^{2+}]_i$, in particular the relative roles of the sarcoplasmic reticulum (SR) and surface membrane are unclear. We investigated the effects on diastolic $[Ca^{2+}]_i$ that result from the changes of Ca cycling known to occur in heart failure. Experiments were performed using Fluo-3 in voltage-clamped rat ventricular myocytes. Increasing stimulation frequency increased diastolic $[Ca^{2+}]_i$. This increase of $[Ca^{2+}]_i$ was larger when SR function was impaired either by making the RyR leaky (with caffeine or ryanodine) or by decreasing SERCA activity with thapsigargin. The increase of diastolic $[Ca^{2+}]_i$ produced by interfering with the SR was accompanied by a decrease of the amplitude of the systolic Ca transient such that there was no change of time-averaged $[Ca^{2+}]_i$. Time-averaged $[Ca^{2+}]_i$ was increased by β-adrenergic stimulation with isoprenaline and increased in a saturating manner with increased stimulation frequency; average $[Ca^{2+}]_i$ was a linear function of Ca entry per unit time. Diastolic and time-averaged $[Ca^{2+}]_i$ were decreased by decreasing the L-type Ca current (with 50 μM cadmium chloride). We conclude that diastolic $[Ca^{2+}]_i$ is controlled by the balance between Ca entry and efflux during systole. Furthermore, manoeuvres which decrease the amplitude of the Ca transient (without decreasing Ca influx) will therefore increase diastolic $[Ca^{2+}]_i$. This identifies a novel mechanism whereby changes of the amplitude of the systolic Ca transient control diastolic $[Ca^{2+}]_i$.

Abbreviations. BDM, 2,3-butanedione monoxime;$[Ca^{2+}]_i$,cytoplasmic Ca ion concentration; ISO, Isoproterenol; NCX, Sodium-Calcium Exchange; RyR – Ryanodine Receptor; SR, Sarcoplasmic Reticulum; SERCA, sarco/endoplasmic reticulum Ca-ATPase

Introduction

For the heart to function as a pump, intracellular Ca concentration ($[Ca^{2+}]_i$) must increase during systole to activate contraction and then fall, during diastole, to levels sufficiently low to allow the myofilaments to relax and the heart to refill with blood. While the mechanisms that control the amplitude of the systolic rise of $[Ca^{2+}]_i$ are well understood (see Bers, 2001 for review), the regulation of the diastolic level is obscure (Louch *et al.*, 2012). One problem is that much work studying $[Ca^{2+}]_i$ has used unphysiologically slow rates of stimulation. Under these conditions, a steady state is reached where Ca entry into the cell balances efflux and there is no flux into and out of the sarcoplasmic reticulum (SR) (Allen *et al.*, 1984;Rios, 2010). In contrast, at faster rates, fluxes of Ca into and out of the SR will play an important role. Indeed, diastolic $[Ca^{2+}]_i$ increases with increasing rate (Layland & Kentish, 1999;Dibb *et al.*, 2007).

An elevation of diastolic $[Ca^{2+}]_i$ has been reported in some studies of heart failure (Gwathmey *et al.*, 1987;Beuckelmann *et al.*, 1992;Sipido *et al.*, 1998;Fischer *et al.*, 2013). The frequency-dependent increase of diastolic $[Ca^{2+}]_i$ (Gwathmey *et al.*, 1991) and force (Pieske *et al.*, 2002) is increased in heart failure in humans and this may contribute to the phenomenon of "diastolic heart failure" (Selby *et al.*, 2011). Raised diastolic $[Ca^{2+}]_i$ also increases Ca leak from the SR by increasing efflux of Ca through the Ryanodine Receptor (RyR) (Bovo *et al.*, 2011) and raised levels due to RyR leak have been suggested to initiate a vicious cycle by further increasing this leak (Louch *et al.*, 2012).

End diastolic $[Ca^{2+}]_i$ presumably depends on a combination of fluxes due to both sarcolemmal and sarcoplasmic reticulum (SR) Ca handling proteins. Heart failure impairs SR function due to a combination of a decrease of SERCA activity and increased RyR leak (see (Lou *et al.*, 2012) for recent review). Previous work has shown that decreasing SERCA

activity elevates diastolic Ca (Negretti *et al.*, 1993). In addition, making the RyR leaky with ryanodine elevates $[Ca^{2+}]_i$, particularly at high stimulation rates (Gao *et al.*, 1995). However, the exact mechanism by which alterations of SR function affect diastolic $[Ca^{2+}]_i$ is unresolved. The aim of the present work was to investigate quantitatively the effects of interfering with the SR on diastolic $[Ca^{2+}]_i$. We find that decreasing SR function decreases systolic and increases diastolic $[Ca^{2+}]_i$. Importantly, there is no effect on the average level of $[Ca^{2+}]_i$. We conclude that the increase of diastolic $[Ca^{2+}]_i$ is a consequence of the decrease of systolic $[Ca^{2+}]_i$ decreasing the efflux of Ca from the cell and, therefore, that systolic $[Ca^{2+}]_i$ plays a major role in controlling diastolic $[Ca^{2+}]_i$.

Methods

Ethical Approval

Care and use of animals were in accordance with The UK Animals (Scientific Procedures) Act, 1986 and Directive 2010/63/EU of the European Parliament. The experiments were approved by the University of Manchester Ethical Review Board. Male Wistar rats (weighing ≈ 200 to 250 g) were killed by stunning and cervical dislocation. Single ventricular myocytes were isolated by digestion with collagenase and protease as described previously (Eisner *et al.*, 1989).

Isolated myocytes were superfused with a solution (control) consisting of (in mM) NaCl 135, Glucose 11.1, $CaCl_2$ 1, Hepes 10, $MgCl_2$ 1, and KCL 4. 4-aminopyridine 5 mM and $BaCl_2$ 0.1 mM were added to inhibit K^+ currents and the solution was titrated to pH 7.4 using NaOH. Probenecid (2 mM) was added to reduce loss of indicator from the myocytes. Micropipettes (<5 MΩ) were filled with a solution consisting of (in mM): KCH_3O_3S 125, KCl 12, NaCl 10, Hepes 10, MgCl2 5, EGTA 0.1; titrated to pH 7.2 with KOH; and a final concentration of amphotericin B of 240 $g.ml^{-1}$. Cells were voltage-clamped with the perforated patch clamp technique using the discontinuous switch clamp mode (frequency 1–2 kHz and gain 0.3–0.7 nA/mV) of an Axoclamp 2A voltage-clamp amplifier (Molecular Devices, Union City, CA, USA). Cells were voltage clamped and stimulated at a range of frequencies (0.2 to 3 Hz) with a 40 mV, 100 ms duration pulse from a holding potential of -40 mV. All experiments were performed at room temperature.

$[Ca^{2+}]_i$ measurements

Cells were incubated with the acetoxymethyl (AM) ester of Fluo-3 (5 μM for 10 minutes) and allowed to de-esterify before use. An aliquot was then placed in a superfusion chamber mounted on the stage of an inverted fluorescence microscope. In order to measure changes of $[Ca^{2+}]_i$, at the end of each experiment the maximum fluorescence (F_{max}) was measured by damaging the cell with the patch pipette. The dissociation constant of Fluo-3 (K_d) was taken as 864 nM (Cheng *et al.*, 1993) and $[Ca^{2+}]_i$ calculated as described previously (Trafford *et al.*, 1999).

Diastolic $[Ca^{2+}]_i$ was calculated by averaging $[Ca^{2+}]_i$ during the final 50 ms before the next stimulus. The amplitude of the Ca transient was calculated by subtracting diastolic

$[Ca^{2+}]_i$ from peak $[Ca^{2+}]_i$. Average $[Ca^{2+}]_i$ was calculated as the mean level from one stimulus to the next. In some experiments SR Ca content was estimated by releasing Ca from the SR using a mixture of 5 mM caffeine and 20 mM 2,3-butanedione monoxime (BDM) (Kashimura *et al.*, 2010).

The Ca influx through the L-type Ca current was calculated by integrating the Ca current (Venetucci *et al.*, 2007). All analysis was performed using custom-written software (Greensmith, 2014).

All chemicals were obtained from Sigma-Aldrich UK, R & D Systems UK or Fisher Scientific UK. Caffeine was added as required. Ryanodine and thapsigargin were both stored as 1 mM stock solutions in DMSO and made up to a concentration of 1 μM before use. Thapsigargin was dissolved in DMSO and stored as a 1 mM stock solution and used at a final concentration of 1 μM.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) where applicable for descriptive analysis. Statistical comparisons were made using two-way ANOVA. The regression lines (Fig. 2D) were compared with a F test. Differences were considered statistically significant when $p < 0.05$.

Results

Effects of RyR leak on diastolic and systolic $[Ca^{2+}]_i$

The first series of experiments examined the effect of increased RyR leak on the response to increased stimulation rate. The grey traces in Fig. 1A show the effect of periods of stimulation at 2 Hz. Under basal conditions the diastolic level of $[Ca^{2+}]_i$ during 2 Hz stimulation was only slightly greater than that during rest. The subsequent addition of caffeine (1 mM) to increase RyR leak decreased the amplitude of the Ca transient and modestly elevated diastolic $[Ca^{2+}]_i$. The effects of β-adrenergic stimulation with isoprenaline (ISO, 1 μM) were then investigated on these phenomena. In agreement with previous work, ISO increased the amplitude of the systolic Ca transient (Hussain & Orchard, 1997); there was little effect on diastolic $[Ca^{2+}]_i$. However, when the cell was stimulated in the presence of caffeine plus ISO, the increase of diastolic $[Ca^{2+}]_i$ was greater than that in caffeine in the absence of ISO. These changes are seen in more detail in the expanded records of Fig. 1B. The origin of the increase of diastolic Ca is revealed in Fig. 1C. Increased leak slows the decay of the Ca transient such that, at elevated rates of stimulation, there is no time for decay to the resting level. In this example the decay is biphasic with a fast phase preceding the slow one. With more extreme leak a slow monophasic decay is seen (Sankaranarayanan *et al.*, 2016). The mean data (Fig. 1D) show values normalized to those obtained at a slow stimulation rate (0.5 Hz) in the absence of both caffeine and ISO. It is clear that the effects of ISO on both systolic and diastolic $[Ca^{2+}]_i$ are greatly affected by the presence of caffeine. For example, in the absence of caffeine, ISO greatly increases the amplitude of the Ca transient (from 82 $\pm$ 3 % to 157 $\pm$ 3 % of control at 2 Hz stimulation, $p<0.001$, ANOVA) while having no effect on diastolic $[Ca^{2+}]_i$ (from 115 $\pm$ 2.5 nM to 121 $\pm$ 3.1 %, $p=0.18$). In contrast, in the presence of caffeine, ISO had a much smaller effect on the amplitude of the Ca transient

(from 36 ± 3 % to 46 ± 3 %, p=0.037) but markedly increased diastolic $[Ca^{2+}]_i$ (from 168 ± 3 % to 208 ± 4 %, p<0.001).

Effects of RyR leak on average $[Ca^{2+}]_i$

The above data show that caffeine increases diastolic $[Ca^{2+}]_i$ while decreasing the systolic rise of $[Ca^{2+}]_i$. Given this, we next investigated the effect of caffeine on time-averaged $[Ca^{2+}]_i$ as shown by the blue trace in Fig. 1A. It is clear that average $[Ca^{2+}]_i$ is (i) increased by stimulation; (ii) increased by ISO; and (iii) unaffected by caffeine in both control and ISO. These observations are confirmed by the mean data of Fig. 1D which show that average $[Ca^{2+}]_i$ does not significantly change upon addition of caffeine (control 151 ± 2 % vs. caffeine 149 + 2 %; p=0.61; data normalized to 0.5 Hz stimulation). A similar finding is seen in the presence of ISO (ISO 208 ± 3 % vs ISO + caffeine 212 ± 4 %; p=0.3). Finally, these data also show that ISO increases average $[Ca^{2+}]_i$ in both the presence and absence of caffeine (p<0.001).

The frequency-dependence of average $[Ca^{2+}]_i$

Since the data of Fig. 1 showed that the effects of caffeine on diastolic $[Ca^{2+}]_i$ were more prominent in the presence of ISO, all subsequent experiments were performed in the presence of ISO. The experiment illustrated in Fig. 2 was designed to investigate Ca handling over a wider range of frequencies. Fig. 2A shows data in the presence and absence of caffeine. As frequency of stimulation increased, the Ca transient amplitude decreased slightly and diastolic $[Ca^{2+}]_i$ increased. The decrease in Ca transient amplitude was accompanied by and is presumably at least in part caused by a decrease in the amplitude of the L-type Ca

current (Fig. 2C) (Antoons *et al.*, 2002;Dibb *et al.*, 2007). The effects of frequency were increased by caffeine. The solid line in Fig. 2A shows, again, that average $[Ca^{2+}]_i$ was unaffected by caffeine and was increased by increasing frequency. Notably, the increase of average $[Ca^{2+}]_i$ was a saturating function of frequency as shown by the fact that raising rate from 0.5 to 1 Hz had a larger effect than that from 2 to 3 Hz.

The above observations of the frequency dependence of average $[Ca^{2+}]_i$ are emphasised by the mean data of Fig. 2B. Caffeine decreased the amplitude of the Ca transient at all frequencies. An increase of frequency increased diastolic $[Ca^{2+}]_i$; this effect is much more obvious in the presence of caffeine. In contrast to the marked effects of caffeine on both diastolic and systolic $[Ca^{2+}]_i$, average $[Ca^{2+}]_i$ was unaffected by caffeine (ANOVA, $p> 0.5$ at all frequencies). Average $[Ca^{2+}]_i$ did, however, increase in a saturating manner with increasing frequency of stimulation. The bottom two panels in Fig. 2B shed light on this saturation of average Ca. The Ca influx via the L-type Ca current on each pulse decreased with increasing stimulation rate (Fig. 2C) due to increasing inactivation (Antoons *et al.*, 2002;Dibb *et al.*, 2007). Consequently the Ca influx per unit time (bottom panel of Fig. 2B) was a saturating function of frequency and therefore paralleled the frequency dependence of average $[Ca^{2+}]_i$. Data obtained in the presence of caffeine (red symbols) were identical to those in its absence (ANOVA, $p>0.5$ at all frequencies). The correlation between Ca entry per unit time and average $[Ca^{2+}]_i$ is emphasised by Fig. 2D which shows a clear linear relationship between these two parameters which is not statistically different (F test; $p=0.064$) in the absence and presence of caffeine.

The effects of thapsigargin and ryanodine

The purpose of the experiments illustrated in Fig. 3&4 was to examine whether the above observations were specific to caffeine or were a general consequence of interfering with RyR function. In the experiment illustrated in Fig. 3A, the application of thapsigargin decreased systolic $[Ca^{2+}]_i$ and increased diastolic $[Ca^{2+}]_i$ during stimulation at 0.5 Hz. On increasing stimulation rate to 2 Hz, there was a more marked increase of diastolic and decrease of systolic $[Ca^{2+}]_i$. When stimulation was stopped $[Ca^{2+}]_i$ declined to a level similar to the original diastolic one. The mean data of Fig. 3B show the effects of thapsigargin at 0.5 and 2 Hz. (Because thapsigargin is irreversible, it was not feasible to study the full range of frequencies used for caffeine). Thapsigargin decreased the amplitude and increased diastolic $[Ca^{2+}]_i$ while having no effect on average $[Ca^{2+}]_i$.

In the experiment illustrated in Fig. 4A, the application of ryanodine decreased systolic $[Ca^{2+}]_i$ and increased diastolic $[Ca^{2+}]_i$. The dark line shows that average $[Ca^{2+}]_i$ remained constant during this period. When stimulation was stopped $[Ca^{2+}]_i$ declined to a level similar to the original diastolic one. Subsequent stimulation at 2 Hz increased average $[Ca^{2+}]_i$. Ryanodine was reapplied to increase its effect. When stimulation was recommenced at 0.5 Hz, the Ca transient was considerably smaller than observed previously at this frequency. The average $[Ca^{2+}]_i$ was, however, virtually identical. Increasing stimulation rate to 2 Hz resulted in a smaller Ca transient than seen at 2 Hz before. Once again, the average Ca was unaffected. These data were interrupted by exposures to caffeine plus BDM to release Ca from the SR and thereby obtain an estimate of the fall of SR Ca content. The mean data of Fig. 4B confirm that ryanodine decreases the amplitude of the Ca transient (to 14.1 $\pm$ 0.7 %, $p<0.001$), increases diastolic $[Ca^{2+}]_i$ (to 273 $\pm$ 3.1 %, $p<0.001$) while having no effect on average $[Ca^{2+}]_i$ (102.8 $\pm$ 1.4 %; $p = 0.17$).

Effects of decreasing Ca influx

The above experiments suggest that the level of diastolic $[Ca^{2+}]_i$ depends on a balance between Ca influx and efflux. If this is the case, one would expect diastolic $[Ca^{2+}]_i$ to be decreased by reducing Ca influx. In the experiment illustrated in Fig. 5A, caffeine had been added while stimulating at either 0.5 or 3 Hz. In agreement with the results above, the rise of diastolic $[Ca^{2+}]_i$ was greater at the higher frequency. Addition of cadmium chloride (50 μM) to decrease the L-type Ca current, decreased diastolic $[Ca^{2+}]_i$ at both stimulation rates (see expanded records of Fig. 5B). On average, cadmium decreased the L-type Ca current to 33 $\pm$ 5 % (n=5 cells). Fig. 5C shows mean data from 5 cells (at 0.5 Hz). One way ANOVA showed that cadmium decreased average and diastolic $[Ca^{2+}]_i$ as well as the amplitude of the Ca transient (all $p<0.001$). Specifically, cadmium decreased average $[Ca^{2+}]_i$ to 69.6 $\pm$ 1.7 %.

Discussion

This paper investigates the control of diastolic $[Ca^{2+}]_i$ in rat ventricular myocytes. In agreement with previous work we find that increasing stimulation frequency increases diastolic $[Ca^{2+}]_i$ (Layland & Kentish, 1999;Dibb *et al.*, 2007). We now add the following, important findings: (i) Diastolic $[Ca^{2+}]_i$ is increased by manoeuvres which decrease SR function such as increased RyR leak or decreased SERCA activity. (ii) This is accompanied by a decrease of systolic $[Ca^{2+}]_i$ such that the time-averaged $[Ca^{2+}]_i$ remains constant. (iii) Time-averaged $[Ca^{2+}]_i$ is increased by β-adrenergic stimulation and is a saturating function of frequency reflecting a frequency dependent decrease of Ca influx per beat. (iv) Diastolic $[Ca^{2+}]_i$ is regulated by the difference between the Ca influx and the systolic efflux on each beat.

The effects of interfering with SR function on diastolic $[Ca^{2+}]_i$

Previous work has shown that ryanodine and thapsigargin increase diastolic $[Ca^{2+}]_i$ (Hansford & Lakatta, 1987;Negretti *et al.*, 1993). In the present paper we elucidated the mechanism of this effect and found that the reduction in Ca transient caused by increasing leak with caffeine or ryanodine was always associated with an increase in diastolic $[Ca^{2+}]_i$. The same behaviour was observed following the application of thapsigargin to inhibit SERCA. In other words, decreased systolic $[Ca^{2+}]_i$ associated with increased diastolic $[Ca^{2+}]_i$ is a distinguishing feature of impaired SR function, no matter whether this results from increased RyR leak or decreased SERCA activity. In the absence of stimulation, interfering with the SR had no effect on the level of resting $[Ca^{2+}]_i$ (Figs 1, 2 & 4). This is to be expected as, under these conditions, $[Ca^{2+}]_i$ is presumably determined solely by the surface membrane

(Allen *et al.*, 1984;Rios, 2010). The increase of diastolic $[Ca^{2+}]_i$ at elevated frequencies results from the fact that the next transient begins before the previous one has relaxed. Increasing leak or decreasing SERCA slows the rate of relaxation of the Ca transient(Negretti *et al.*, 1993;Belevych *et al.*, 2007;Sankaranarayanan *et al.*, 2016). A combination of increased frequency and slowed relaxation would be expected to elevate diastolic $[Ca^{2+}]_i$. The frequency-dependence of diastolic $[Ca^{2+}]_i$ is particularly obvious for thapsigargin (Fig 3B). This may be because inhibition of SERCA greatly slows the decay of the Ca transient. At low rates the increased duration of the Ca transient compensates for the decreased amplitude in maintaining efflux such that diastolic $[Ca^{2+}]_i$ does not increase. This is impossible at high rates as the decay of the transient is interrupted by the next stimulus.

A key question is what causes the inverse relationship between diastolic and systolic $[Ca^{2+}]_i$ such that average $[Ca^{2+}]_i$ is maintained constant when SR function is altered? We suggest that the answer resides in the mechanisms that maintain Ca flux balance. In the steady state, during each cycle of stimulation, the influx mediated by the Ca current must be precisely equal to the Ca efflux, largely via NCX (see Eisner *et al.,*2013 (2013) for review). The activity of NCX depends on $[Ca^{2+}]_i$. Increasing SR leak or decreasing SERCA activity will decrease the SR Ca content and therefore the amplitude of the systolic Ca transient. This, in turn, will decrease Ca efflux to a level less than the influx. Consequently, the cell will gain Ca and, since the SR is compromised, much of this Ca will remain in the cytoplasm, increasing diastolic $[Ca^{2+}]_i$. This elevated diastolic $[Ca^{2+}]_i$ produces more Ca efflux and compensates for the loss of efflux associated with the systolic transient (Dibb *et al.*, 2007). If we assume that NCX activity is proportional to $[Ca^{2+}]_i$ then the Ca efflux per cycle will be proportional to average $[Ca^{2+}]_i$. If Ca influx is unaffected then the need for constant efflux requires that average $[Ca^{2+}]_i$ be constant and therefore the decrease of systolic $[Ca^{2+}]_i$ must be balanced by an increase of diastolic such that average $[Ca^{2+}]_i$ remains constant.

This consideration of flux balance is a more complicated and general version of previous work showing that potentiating the opening of the RyR with low concentrations of caffeine leads to a transient increase of the amplitude of the systolic Ca transient (Trafford *et al.*, 2000;Greensmith *et al.*, 2014). In the steady state, however, the amplitude of the Ca transient was the same as in control. Under the conditions of those experiments (performed at low rates of stimulation), diastolic $[Ca^{2+}]_i$ did not change and therefore maintenance of flux balance required that systolic $[Ca^{2+}]_i$ was constant. In the present experiments, the changes of systolic $[Ca^{2+}]_i$ required that diastolic $[Ca^{2+}]_i$ change in order to maintain flux balance.

The effects of β-adrenergic stimulation on $[Ca^{2+}]_i$

The above analysis also explains why ISO increases average $[Ca^{2+}]_i$. ISO will increase Ca entry through the L-type current and this will have to be balanced by increased efflux on NCX. This increased efflux can be achieved by an increase of average $[Ca^{2+}]_i$. The exact circumstances will determine whether the increase of average $[Ca^{2+}]_i$ results primarily from a rise of diastolic as opposed to systolic $[Ca^{2+}]_i$. For example (Fig. 1), with normal SR function, the increase of the amplitude of the systolic Ca transient is sufficiently large that diastolic $[Ca^{2+}]_i$ does not increase. In contrast when the SR is partly disabled, systolic $[Ca^{2+}]_i$ cannot increase sufficiently and a rise of diastolic $[Ca^{2+}]_i$ ensues (Fig. 1D). It is also possible that an increase of Ca leak from the SR, possibly via a Ca Calmodulin dependent kinase II (CaMKII) mechanism (Curran *et al.*, 2007), contributes to the increase of diastolic $[Ca^{2+}]_i$. In this context it is worth noting that in mice overexpressing CaMKII, the rise of systolic $[Ca^{2+}]_i$ produced by ISO was less than in wild type. However ISO produced a larger increase of diastolic $[Ca^{2+}]_i$ in the overexpressing mice compared to control (Sag *et al.*, 2009). Given that these transgenic mice have elevated SR Ca leak, the reciprocal effect on diastolic and systolic

$[Ca^{2+}]_i$ is consistent with the conclusions of our work. It should also be noted that the normal inotropic response to ISO, resulting from an increase of systolic $[Ca^{2+}]_i$ with no change of diastolic, requires a normal, functional SR. As SR activity is compromised an increase of diastolic $[Ca^{2+}]_i$ will occur.

The effects of stimulation frequency

An increase of stimulation frequency will increase Ca influx per unit time thereby requiring an increase of average $[Ca^{2+}]_i$ to maintain flux balance. From first principles this can be achieved by an increase of either or both diastolic or systolic $[Ca^{2+}]_i$. In the present experiments, performed on rat myocytes, increased frequency decreases systolic $[Ca^{2+}]_i$ and therefore systolic efflux. As frequency is increased, the cell therefore faces three challenges: (i) increased Ca influx per unit time (Fig. 2B); (ii) decreased systolic efflux; (iii) decreased diastolic time for efflux to occur in. Ca flux balance can therefore only be established with an increase of diastolic $[Ca^{2+}]_i$. If SR function is depressed, increased frequency produces a larger decrease of systolic $[Ca^{2+}]_i$ and one would therefore expect a larger rise of diastolic $[Ca^{2+}]_i$. In agreement with these predictions, we find that increasing frequency increases diastolic $[Ca^{2+}]_i$ and this increase is potentiated by increasing SR leak or decreasing SERCA activity. Average $[Ca^{2+}]_i$ is a saturating function of frequency (Fig. 2B) presumably because the Ca entry per unit time also saturates with frequency due to increased inactivation of the L-type Ca current (Fig. 2C), (Antoons *et al.*, 2002;Dibb *et al.*, 2007). Consistent with this, average $[Ca^{2+}]_i$ is a linear function of Ca influx per unit time through the L-type Ca current (Fig. 2D). It should be noted that if the L-type Ca current did not decrease at higher frequencies the rise of diastolic $[Ca^{2+}]_i$ would be even greater.

One final conclusion can be derived from Fig. 2D. It is clear that, even with zero influx through the L-type Ca current, $[Ca^{2+}]_i$ has a finite value. This has been accounted for by a background Ca entry (Choi *et al.*, 2000;Kupittayanant *et al.*, 2006) which is unaffected by stimulation rate. The value of this background flux, estimated from the horizontal intercept of Fig. 2D is of the order of 4 $\mu mol.l^{-1}.s^{-1}$. The existence of this background flux may also be relevant to the effects of cadmium. We found that 50 μM Cd decreased Ca influx to 33% but average $[Ca^{2+}]_i$ fell to only 70%. This discrepancy can be accounted for if the background flux is unaffected by Cd.

Limitations

It should be noted that in these experiments we used a holding potential of -40 mV to inactivate the Na^+ current. This holding potential will decrease the L-type Ca current and lead to an underestimate of the effects of frequency on diastolic $[Ca^{2+}]_i$ (Dibb *et al.*, 2007). The removal of Na^+ current might be expected to decrease the frequency-dependent increase of intracellular Na^+ concentration but since Na^+ influx through Na channels is quantitatively smaller than that through NCX (Bers *et al.*, 2003), this may not be a major issue. Previous work has measured systolic and diastolic $[Ca^{2+}]_i$ in rat ventricular myocytes excited with physiological action potentials. An increase of frequency increased both diastolic and systolic $[Ca^{2+}]_i$ indicated that a frequency-dependent increase of average $[Ca^{2+}]_i$ is also seen with more physiological stimulation (Dibb *et al.*, 2007). A more general point is that the above discussion assumes that the only factor regulating NCX is $[Ca^{2+}]_i$. It therefore ignores the effects that changes of intracellular sodium concentration ($[Na^+]_i$) may have on NCX activity. $[Na^+]_i$ will be increased by an increase of stimulation rate and as a consequence of the increased NCX activity in response to the Ca loading produced by β-adrenergic stimulation.

It will be decreased as a consequence of phosphorylation of phospholemman and stimulation of the sodium pump (see Bers *et al.*, 2003 for a recent review). It should also be noted that the above discussion is based on the assumption that NCX activity is proportional to $[Ca^{2+}]_i$. This will only be true over a certain range and, at higher $[Ca^{2+}]_i$, efflux will tend towards saturation. In this case, when SR function is decreased one might expect a reduction of average $[Ca^{2+}]_i$. Any such effect is below the resolution of the experiments. Finally, although in the present experiments Ca influx through the L-type current was unaffected by altering SR function with caffeine, more generally it is possible that changes of Ca-dependent inactivation may affect the L-type Ca current and this would need to be allowed for,

Relationship to disease

Some previous work has shown that heart failure results in an increase of diastolic force and/or $[Ca^{2+}]_i$ at elevated rates of stimulation (Sipido *et al.*, 1998;Baartscheer *et al.*, 2003;Selby *et al.*, 2011). One explanation for this is the measured increase of $[Na^+]_i$ which will decrease Ca efflux on NCX (Pieske *et al.*, 2002). The results of the present paper suggest an additional explanation for the rise of diastolic $[Ca^{2+}]_i$. It is known that heart failure is often associated with increased RyR leak (Marks, 2000;Marx *et al.*, 2000;Shannon *et al.*, 2003;Terentyev *et al.*, 2008;Belevych *et al.*, 2013) and decreased SERCA activity (Nagai *et al.*, 1989;Mercadier *et al.*, 1990;Hasenfuss *et al.*, 1994). As a consequence of the resulting decrease of SR Ca content and thence systolic $[Ca^{2+}]_i$, these changes would be expected to also elevate diastolic $[Ca^{2+}]_i$. Indeed both this mechanism and the increase of $[Na^+]_i$ will decrease Ca efflux and increase diastolic $[Ca^{2+}]_i$.

A common observation is that, in human heart failure, the reduction of the amplitude of the Ca transient is more marked at higher frequencies (Gwathmey *et al.*, 1990;Mulieri *et al.*, 1992). This is accompanied by removal of the increase of SR Ca content produced by

increasing stimulation frequency (Lindner *et al.*, 1998;Pieske *et al.*, 1999). These effects have previously been attributed to a decrease of SERCA activity (Pieske *et al.*, 1995). Our data suggest that a Ca leak may also contribute to the loss of this frequency dependence in heart failure. The results of this paper are also relevant to the changes produced by increasing Ca buffering by the myofilaments as occurs in some case of hypertrophic cardiomyopathy (HCM). Such increased buffering slows the rate constant of decay of the Ca transient and increases diastolic $[Ca^{2+}]_i$ (Schober *et al.*, 2012). The increased buffering will decrease the increase of $[Ca^{2+}]_i$ produced by a given total Ca release from the SR, thereby decreasing Ca efflux. This, and the slowed decay will elevate diastolic $[Ca^{2+}]_i$.

Conclusion: Systolic $[Ca^{2+}]_i$ *controls diastolic*

The results of this paper show that the time-averaged level of $[Ca^{2+}]_i$ is an important factor in regulating Ca cycling. This average level determines the Ca efflux from the cell required to balance Ca influx. The total efflux can be thought of as comprising two components: (i) that activated by the diastolic level of $[Ca^{2+}]_i$ and (ii) an additional component that occurs during the systolic Ca transient (Dibb *et al.*, 2007). Anything which decreases the amplitude of the systolic Ca transient, without affecting Ca influx will decrease the systolic efflux thereby requiring an increase of diastolic $[Ca^{2+}]_i$ to maintain Ca flux balance. Seen in this way, the systolic Ca transient plays a vital role in regulating diastolic $[Ca^{2+}]_i$.

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Competing Interests

None

Author Contributions

DAE and LV designed the work. RS and KK performed the experiments and, with DJG, analysed and interpreted the data. DAE, RS and LV wrote the manuscript and all authors revised it critically for important intellectual content. All authors approved the final version of the manuscript. All the experiments were carried out at The University of Manchester.

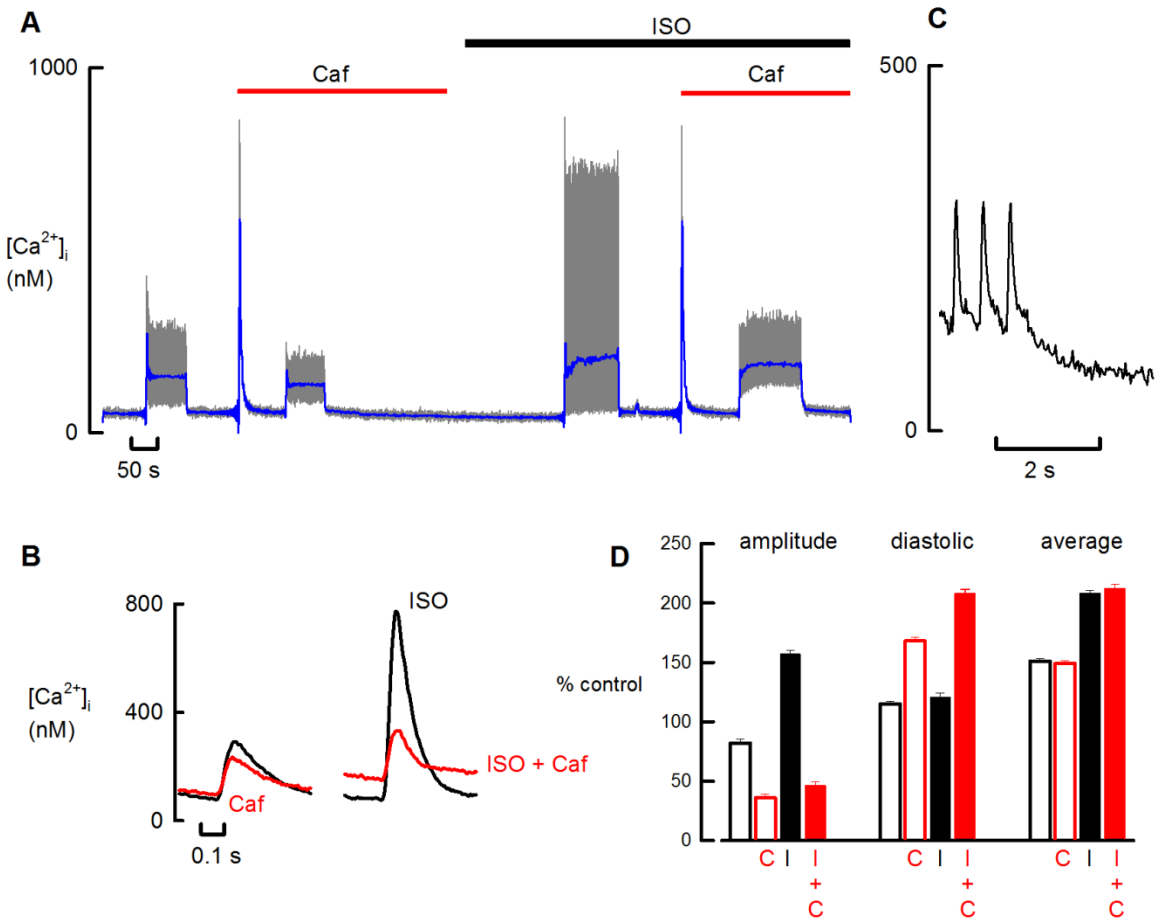

Fig. 1. The effects of caffeine on systolic, diastolic and average $[Ca^{2+}]_i$. A. Original timecourse. The grey trace shows $[Ca^{2+}]_i$ during rest and stimulation at 2 Hz. Caffeine (Caf, 1 mM) and isoprenaline (ISO, 1 μM) were applied as shown. The blue trace shows the average $[Ca^{2+}]_i$ for each transient. **B.** Specimen, averaged (n=20) transients. The left hand records were obtained in the absence and the right hand ones in the presence of ISO. Red traces obtained in the presence of caffeine (1 mM). **C**. Decay of $[Ca^{2+}]_i$ at the end of stimulation. The record shows the last three transients and the period afterwards from A in ISO + Caf. **D**. Mean data from 6 experiments. Bars show data at a stimulation rate of 2 Hz. For each cell, the data have been normalized to the corresponding values obtained during stimulation at 0.5 Hz in the absence of both caffeine and ISO. The left-hand group of 4 bars shows amplitude, the next group diastolic $[Ca^{2+}]_i$ and the right hand average $[Ca^{2+}]_i$. In each group the unlabelled bar is control; C, caffeine; I, ISO; I+C, ISO + caffeine. For clarity, statistical significance is not presented on the figure but is reported in the main text.

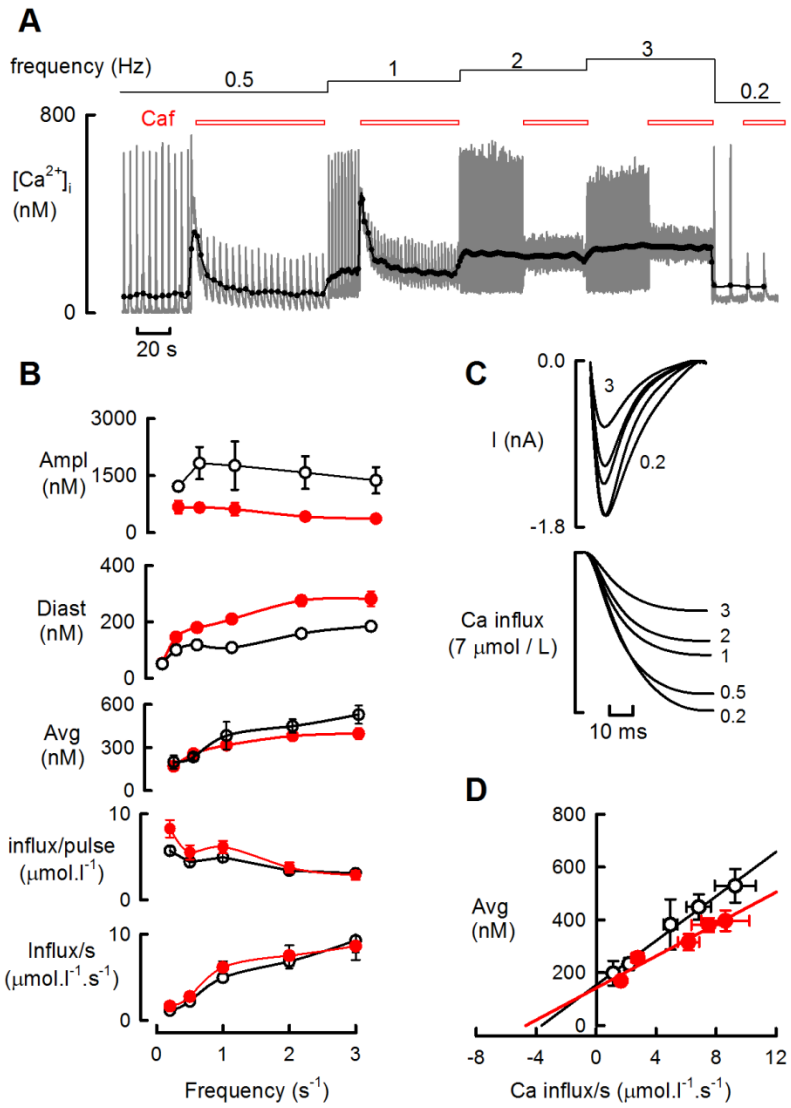

Fig. 2. The frequency dependence of the effects of interfering with SR function on $[Ca^{2+}]_i$. A. Timecourse of effects of caffeine. Stimulation frequency was altered and caffeine (Caf, 1 mM) applied as shown above. ISO (1 μM) was present throughout. The grey trace shows the original data and the dark one the average $[Ca^{2+}]_i$ on each transient. **B**. Mean data showing frequency dependence**.** Panels show mean data (14 to 20 cells) of the effects of frequency on the following parameters (from top to bottom): amplitude of the Ca transient; diastolic $[Ca^{2+}]_i$; average $[Ca^{2+}]_i$; Ca influx per pulse; Ca influx per second. In all panels, red symbols in presence of caffeine. **C**. Effects of stimulation frequency on the L-type Ca current (absence of caffeine). Top, specimen Ca currents obtained at frequencies from 0.2 to 3 Hz. Bottom, timecourse of the integral of the Ca current to show Ca influx. For clarity, the full range of frequencies is only indicated on the integral traces. For the Ca currents, the extreme frequencies are indicated. For the other frequencies, the order of speed of inactivation of the L-type Ca current parallels simulation frequency. **D.** Relationship between average $[Ca^{2+}]_i$ and Ca influx per second. Data taken from **B**. Black symbols show absence and red presence of caffeine.

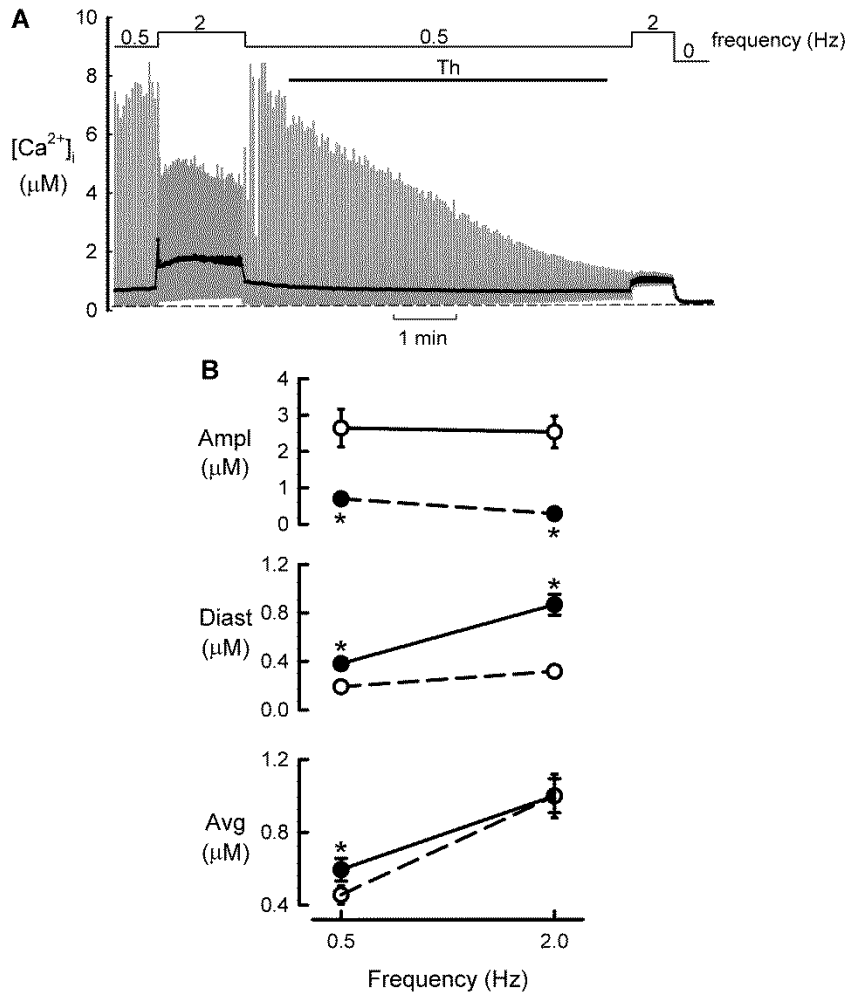

Fig. 3. The effects of interfering with SR Ca handling with thapsigargin on $[Ca^{2+}]_i$ during stimulation. All experiments were performed in the presence of ISO (1 μM). **A.** Effects of thapsigargin. Thapsigargin (Th, 1 μM) were applied for the period shown above. The cell was stimulated at the frequencies indicated below. **B**. Mean data from 10-12 experiments at stimulus rates of 0.5 and 2 Hz. Panels show (from top to bottom): amplitude; diastolic; and average $[Ca^{2+}]_i$. Open symbols before and closed in presence of thapsigargin. * $p<0.05$ (two way repeated measures ANOVA) for comparisons between control and thapsigargin. (ANOVA performed on the 9 cells which had measurements at both frequencies.)

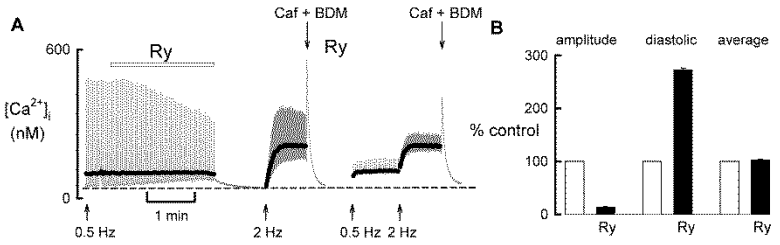

Fig. 4. The effects of ryanodine. A. Timecourse of the effects of ryanodine (1 μM) on $[Ca^{2+}]_i$ studied at rest, 0.5 and 2 Hz. **B.** Mean data from 6 experiments. Bars show data at a stimulation rate of 2 Hz. For each cell, the data have been normalized to the corresponding values obtained during stimulation in the absence of ryanodine. The left hand two bars show amplitude, the next diastolic $[Ca^{2+}]_i$ and the right hand average $[Ca^{2+}]_i$. In each group the unlabelled, open bar is control and the filled is ryanodine (Ry).

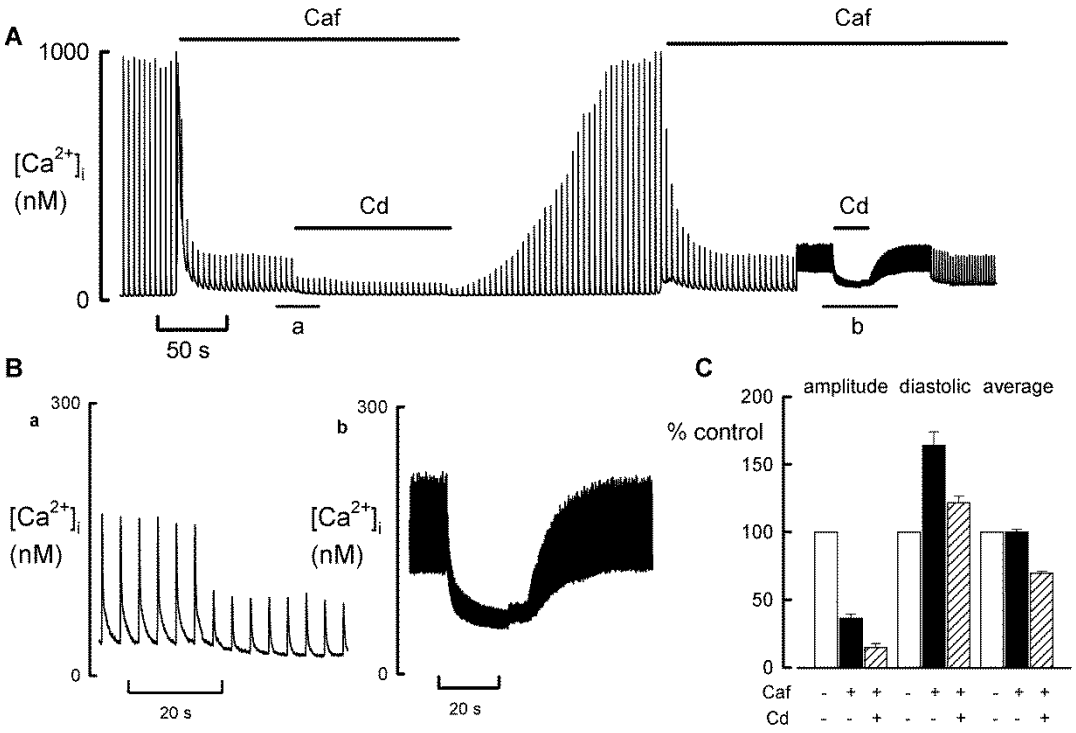

Fig. 5. Effects of cadmium on diastolic $[Ca^{2+}]_i$. A. Time course. The cell had been exposed to ISO (1 μM). Caffeine (Caf, 1 mM) and cadmium (Cd, 50 μM) were applied for the periods shown. Stimulation rate was 0.5 Hz until it was elevated to 3 Hz as shown. **B**. Expanded records of the periods of application of Cd during stimulation at 0.5 (**a**) and 3 (**b**) Hz. **C**. Mean (n= 5 cells) data showing effects of Caf alone and Caf + Cd on amplitude, diastolic and average $[Ca^{2+}]_i$ at 0.5 Hz.

Reference List

Allen DG, Eisner DA, & Orchard CH (1984). Factors influencing free intracellular calcium concentration in quiescent ferret ventricular muscle. *J Physiol (Lond)* **350**, 615-630.

Antoons G, Mubagwa K, Nevelsteen I, & Sipido KR (2002). Mechanisms underlying the frequency dependence of contraction and Ca^{2+} transients in mouse ventricular myocytes. *J Physiol (Lond)* **543**, 889-898.

Baartscheer A, Schumacher CA, Belterman CNW, Coronel R, & Fiolet JWT (2003). SR calcium handling and calcium after-transients in a rabbit model of heart failure. *Cardiovasc Res* **58**, 99-108.

Belevych A, Kubalova Z, Terentyev D, Hamlin RL, Carnes CA, & Györke S (2007). Enhanced ryanodine receptor-mediated calcium leak determines reduced sarcoplasmic reticulum calcium content in chronic canine heart failure. *Biophys J* **93**, 4083-4092.

Belevych AE, Radwanski PB, Carnes CA, & Gyorke S (2013). 'Ryanopathy': causes and manifestations of RyR2 dysfunction in heart failure. *Cardiovasc Res* **98**, 240-247.

Bers DM (2001). *Excitation-Contraction Coupling and Cardiac Contractile Force.*, 2 ed. Kluwer Academic Publishers, Dordrecht/Boston/London.

Bers DM, Barry WH, & Despa S (2003). Intracellular Na^+ regulation in cardiac myocytes. *Cardiovasc Res* **57**, 897-912.

Beuckelmann DJ, Näbauer M, & Erdmann E (1992). Intracellular Calcium Handling in Isolated Ventricular Myocytes From Patients with Terminal Heart Failure. *Circulation* **85**, 1046-1055.

Bovo E, Mazurek SR, Blatter LA, & Zima AV (2011). Regulation of sarcoplasmic reticulum Ca2+ leak by cytosolic Ca2+ in rabbit ventricular myocytes. *The Journal of Physiology* **589**, 6039-6050.

Cheng H, Lederer WJ, & Cannell MB (1993). Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262**, 740-744.

Choi HS, Trafford AW, & Eisner DA (2000). Measurement of calcium entry and exit in quiescent rat ventricular myocytes. *Pflügers Archiv* **440**, 600-608.

Curran J, Hinton MJ, Rios E, Bers DM, & Shannon TR (2007). Beta-adrenergic enhancement of sarcoplasmic reticulum calcium leak in cardiac myocytes is mediated by calcium/calmodulin-dependent protein kinase. *Circ Res* **100**, 391-398.

Dibb KM, Eisner DA, & Trafford AW (2007). Regulation of systolic $[Ca^{2+}]_i$ and cellular Ca^{2+} flux balance in rat ventricular myocytes by SR Ca^{2+}, L-type Ca^{2+} current and diastolic $[Ca^{2+}]_i$. *J Physiol (Lond)* **585**, 579-592.

Eisner D, Bode E, Venetucci L, & Trafford A (2013). Calcium flux balance in the heart. *J Mol Cell Cardiol* **58**, 110-117.

Eisner DA, Nichols CG, O'Neill SC, Smith GL, & Valdeolmillos M (1989). The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J Physiol (Lond)* **411**, 393-418.

Fischer TH, Herting J, Tirilomis T, Renner A, Neef S, Toischer K, Ellenberger D, Forster A, Schmitto JD, Gummert J, Schondube FA, Hasenfuss G, Maier LS, & Sossalla S (2013). CaMKII and PKA differentially regulate SR Ca^{2+}-leak in human cardiac pathology. *Circulation* **128**, 970-981.

Gao WD, Atar D, Backx PH, & Marban E (1995). Relationship between intracellular calcium and contractile force in stunned myocardium. Direct evidence for decreased myofilament Ca2+ responsiveness and altered diastolic function in intact ventricular muscle. *Circ Res* **76**, 1036-1048.

Greensmith DJ (2014). Ca analysis: an Excel based program for the analysis of intracellular calcium transients including multiple, simultaneous regression analysis. *Comput Methods Programs Biomed* **113**, 241-250.

Greensmith DJ, Galli G, Trafford AW, & Eisner DA (2014). Direct measurements of SR free Ca reveal the mechanism underlying the transient effects of RyR potentiation under physiological conditions. *Cardiovasc Res* **103**, 554-563.

Gwathmey JK, Copelas L, MacKinnon R, Schoen FJ, Feldman MD, Grossman W, & Morgan JP (1987). Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. *Circ Res* **61**, 70-76.

Gwathmey JK, Slawsky MT, Hajjar RJ, Briggs GM, & Morgan JP (1990). Role of intracellular calcium handling in force-interval relationships of human ventricular myocardium. *J Clin Invest* **85**, 1599-1613.

Gwathmey JK, Warren SE, Briggs GM, Copelas L, Feldman MD, Phillips PJ, Callahan MJr, Schoen FJ, Grossman W, & Morgan JP (1991). Diastolic dysfunction in hypertrophic cardiomyopathy. Effect on active force generation during systole. *J Clin Invest* **87**, 1023-1031.

Hansford RG & Lakatta EG (1987). Ryanodine releases calcium from sarcoplasmic reticulum in calcium-tolerant rat cardiac myocytes. *J Physiol (Lond)* **390**, 453-467.

Hasenfuss G, Reinecke H, Studer R, Meyer M, Pieske B, Holtz J, Holubarsch C, Posival H, Just H, & Drexler H (1994). Relation between myocardial function and expression of sarcoplasmic reticulum Ca^{2+}-ATPase in failing and nonfailing human myocardium. *Circ Res* **75**, 434-442.

Hussain M & Orchard CH (1997). Sarcoplasmic reticulum Ca^{2+} content, L-type Ca^{2+} current and the Ca^{2+} transient in rat myocytes during β-adrenergic stimulation. *J Physiol (Lond)* **505**, 385-402.

Kashimura T, Briston SJ, Trafford AW, Napolitano C, Priori SG, Eisner DA, & Venetucci LA (2010). In the RyR2R4496C mouse model of CPVT, β-adrenergic stimulation induces Ca waves by increasing SR Ca content and not by decreasing the threshold for Ca waves. *Circ Res* **107**, 1483-1489.

Kupittayanant P, Trafford AW, Diaz ME, & Eisner DA (2006). A mechanism distinct from the L-type Ca current or Na-Ca exchange contributes to Ca entry in rat ventricular myocytes. *Cell Calcium* **39**, 417-423.

Layland J & Kentish JC (1999). Positive force- and $[Ca^{2+}]_i$-frequency relationships in rat ventricular trabeculae at physiological frequencies. *Am J Physiol* **276**, H9-18.

Lindner M, Erdmann E, & Beuckelmann DJ (1998). Calcium content of the sarcoplasmic reticulum in isolated ventricular myocytes from patients with terminal heart failure. *J Mol Cell Cardiol* **30**, 743-749.

Lou Q, Janardhan A, & Efimov I (2012). Remodeling of Calcium Handling in Human Heart Failure. In *Calcium Signaling*, ed. Islam M, pp. 1145-1174. Springer Netherlands.

Louch WE, Stokke MK, Sjaastad I, Christensen G, & Sejersted OM (2012). No Rest for the Weary: Diastolic Calcium Homeostasis in the Normal and Failing Myocardium. *Physiology* **27**, 308-323.

Marks AR (2000). Cardiac intracellular calcium release channels role in heart failure. *Circ Res* **87**, 8-11.

Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, & Marks AR (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365-376.

Mercadier JJ, Lompre AM, Duc P, Boheler KR, Fraysse JB, Wisnewsky C, Allen PD, Komajda M, & Schwartz K (1990). Altered sarcoplasmic reticulum Ca^{2+}-ATPase gene expression in the human ventricle during end-stage heart failure. *J Clin Invest* **85**, 305-309.

Mulieri LA, Hasenfuss G, Leavitt B, Allen PD, & Alpert NR (1992). Altered myocardial force-frequency relation in human heart failure. *Circulation* **85**, 1743-1750.

Nagai R, Zarain-Herzberg A, Brandl CJ, Fujii J, Tada M, MacLennan DH, Alpert NR, & Periasamy M (1989). Regulation of myocardial Ca^{2+}-ATPase and phospholamban mRNA expression in response to pressure overload and thyroid hormone. *PNAS* **86**, 2966-2970.

Negretti N, O'Neill SC, & Eisner DA (1993). The effects of inhibitors of sarcoplasmic reticulum function on the systolic Ca^{2+} transient in rat ventricular myocytes. *J Physiol (Lond)* **468**, 35-52.

Pieske B, Kretschmann B, Meyer M, Holubarsch C, Weirich J, Posival H, Minami K, Just H, & Hasenfuss G (1995). Alterations in intracellular calcium handling associated with the inverse force-frequency relation in human dilated cardiomyopathy. *Circulation* **92**, 1169-1178.

Pieske B, Maier LS, Bers DM, & Hasenfuss G (1999). Ca^{2+} handling and sarcoplasmic reticulum Ca^{2+} content in isolated failing and nonfailing human myocardium. *Circ Res* **85**, 38-46.

Pieske B, Maier LS, Piacentino V, III, Weisser J, Hasenfuss G, & Houser S (2002). Rate Dependence of [Na+]i and Contractility in Nonfailing and Failing Human Myocardium. *Circulation* **106**, 447-453.

Rios E (2010). The cell boundary theorem: a simple law of the control of cytosolic calcium concentration. *J Physiol Sci* **60**, 81-84.

Sag CM, Wadsack DP, Khabbazzadeh S, Abesser M, Grefe C, Neumann K, Opiela MK, Backs J, Olson EN, Brown JH, Neef S, Maier SKG, & Maier LS (2009). Calcium/Calmodulin-Dependent Protein Kinase II Contributes to Cardiac Arrhythmogenesis in Heart Failure. *Circulation Heart Failure* **2**, 664-675.

Sankaranarayanan R, Li Y, Greensmith DJ, Eisner DA, & Venetucci L (2016). Biphasic decay of the Ca transient results from increased sarcoplasmic reticulum Ca leak. *J Physiol* **594**, 611-623.

Schober T, Huke S, Venkataraman R, Gryshchenko O, Kryshtal D, Hwang HS, Baudenbacher FJ, & Knollmann BC (2012). Myofilament Ca Sensitization Increases Cytosolic Ca Binding Affinity, Alters Intracellular Ca Homeostasis, and Causes Pause-Dependent Ca-Triggered Arrhythmia. *Circ Res* **111**, 170-179.

Selby DE, Palmer BM, LeWinter MM, & Meyer M (2011). Tachycardia-induced diastolic dysfunction and resting tone in myocardium from patients with a normal ejection fraction. *J Am Coll Cardiol* **58**, 147-154.

Shannon TR, Pogwizd SM, & Bers DM (2003). Elevated Sarcoplasmic Reticulum Ca^{2+} Leak in Intact Ventricular Myocytes From Rabbits in Heart Failure. *Circ Res* **93**, 592-594.

Sipido KR, Stankovicova T, Flameng W, Vanhaecke J, & Verdonck F (1998). Frequency dependence of Ca^{2+} release from the sarcoplasmic reticulum in human ventricular myocytes from end-stage heart failure. *Cardiovasc Res* **37**, 478-488.

Terentyev D, Gyorke I, Belevych AE, Terentyeva R, Sridhar A, Nishijima Y, de Blanco EC, Khanna S, Sen CK, Cardounel AJ, Carnes CA, & Gyorke S (2008). Redox modification of ryanodine receptors contributes to sarcoplasmic reticulum Ca^{2+} leak in chronic heart failure. *Circ Res* **103**, 1466-1472.

Trafford AW, Díaz ME, & Eisner DA (1999). A novel, rapid and reversible method to measure Ca buffering and timecourse of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes. *Pflügers Archiv* **437**, 501-503.

Trafford AW, Díaz ME, Sibbring GC, & Eisner DA (2000). Modulation of CICR has no maintained effect on systolic Ca^{2+}: simultaneous measurements of sarcoplasmic reticulum and sarcolemmal Ca^{2+} fluxes in rat ventricular myocytes. *J Physiol (Lond)* **522**, 259-270.

Venetucci L, Trafford AW, & Eisner DA (2007). Increasing ryanodine receptor open probability alone does not produce arrhythmogenic Ca^{2+} waves: threshold Ca^{2+} content is required. *Circ Res* **100**, 105-111.