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Changes of SE activity have only modest effects on sarcoplasmic r lum Ca^{2+} content in rat ventricular myocytes

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Non-technical summary Cardiac contraction is caused by an increase of calcium (Ca^{2+}) concentration in the cells of the heart, the so-called 'systolic Ca^{2+} transient'. The majority of this Ca^{2+} is provided by the sarcoplasmic reticulum (SR), which acts as a Ca^{2+} store within the cell itself. Before the heart can contract again the Ca^{2+} store needs to be replenished and so Ca^{2+} is pumped back into the SR by the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA). We show that a given fractional decrease of SERCA activity produces a much smaller decrease of SR Ca^{2+} content. This means that changes of SERCA activity can produce large changes of systolic Ca^{2+} without the need for energetically expensive alterations of SR Ca^{2+} content.

Abstract Changes of the activity of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) affect the amplitude of the systolic Ca^{2+} transient and thence cardiac contractility. This is thought to be due to alterations of SR Ca^{2+} content. Recent work on mice in which the expression of SERCA is decreased found that a large reduction of SERCA expression resulted in a proportionately much smaller decrease of SR Ca^{2+} content. The aim of the current work was to investigate the quantitative nature of the dependence of both the amplitude of the systolic Ca^{2+} transient and SR Ca^{2+} content on SERCA activity during acute partial inhibition of SERCA. Experiments were performed on rat ventricular myocytes. Brief application of thapsigargin (1 μM) resulted in a decrease of SERCA activity as measured from the rate of decay of the systolic Ca^{2+} transient. This was accompanied by a decrease in the amplitude of the systolic Ca^{2+} transient which was linearly related to that of SERCA activity. However, the fractional decrease in the SR Ca^{2+} content was much less than that of SERCA activity. On average SR Ca^{2+} content was proportional to SERCA activity raised to the 0.38 ± 0.07 power. This shallow dependence of SR content on SERCA activity arises because Ca^{2+} release is a steep function of SR Ca^{2+} content. In contrast SR Ca^{2+} content was increased 4.59 ± 0.40 ($n = 8$)-fold by decreasing ryanodine receptor opening with tetracaine (1 mM). Therefore a modest decrease of SR Ca^{2+} content results in a proportionately larger fall of Ca^{2+} release from the SR which can balance a larger initiating decrease of SERCA. In conclusion, the shallow dependence of SR Ca^{2+} content on SERCA activity is expected for a system in which small changes of SR Ca^{2+} content produce larger effects on the amplitude of the systolic Ca^{2+} transient.

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Abbreviations BDM, 2,3-butanedione monoxime; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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Introduction

The sarcoplasmic reticulum (SR) is fundamental to Ca^{2+} handling in cardiac muscle. Most of the Ca^{2+} that activates contraction comes from the SR and is released during systole by the process of Ca^{2+} induced Ca^{2+} release. Ca^{2+} leaves the SR through a release channel (known as the ryanodine receptor, RyR). Ca^{2+} release from the SR is triggered by an increase of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by the L-type Ca^{2+} current (see Bers, 2002 for review). Ca^{2+} release is also sensitive to the intra-SR ('luminal') Ca^{2+} concentration with Ca^{2+} release being a steep function of SR Ca^{2+} concentration (Bassani *et al.* 1995; Trafford *et al.* 1997, 2000). In other words the amplitude of the systolic Ca^{2+} transient and thence contractility is regulated by SR Ca^{2+} content.

A major factor that determines SR Ca^{2+} content is the activity of the SR Ca^{2+}-ATPase (SERCA). An increase of SERCA activity would be expected to increase SR Ca^{2+} content and thereby increase cardiac contractility. Consistent with this, changes of SERCA activity are involved in both normal and pathological changes of cardiac Ca^{2+} handling. SERCA is regulated by an endogenous inhibitor, phospholamban. During β-adrenergic receptor stimulation, phospholamban is phosphorylated and SERCA activity increases (Tada *et al.* 1974). There is also abundant evidence that in heart failure SERCA activity is decreased and this contributes to the observed decrease of SR Ca^{2+} content and contractility (Hasenfuss *et al.* 1994; Hobai & O'Rourke, 2001; Pogwizd *et al.* 2001; Díaz *et al.* 2004; Briston *et al.* 2011). Indeed viral transfer of SERCA is being investigated as a means of treating heart failure (Kawase *et al.* 2011).

Given the importance of SERCA as outlined above, there has been much interest in examining the effects of knocking out SERCA. Complete knockout is embryonically lethal (Periasamy *et al.* 1999). Recently, however, mice have been bred in which SERCA can be conditionally knocked out (Andersson *et al.* 2009; Stokke *et al.* 2010). In these mice, even when cardiac SERCA expression was reduced to less than 5% of control, the animals continued to live for 6–8 weeks (Andersson *et al.* 2009). Furthermore, despite the reduction of SERCA to less than 5% of control levels, the SR Ca^{2+} content fell to only 38% of control values. In other words a very large fractional decrease of SERCA activity is associated with a much smaller fractional decrease of total SR Ca^{2+} content.

A major aim of the present paper is to investigate the mechanism responsible for this relative lack of sensitivity of SR Ca^{2+} content to SERCA activity. One concern with studies using genetic manipulation is that compensatory changes may occur. We therefore decided to investigate the effects of specific partial pharmacological inhibition of SERCA using thapsigargin. The results show that this also produces a non-linear relationship between SERCA activity and SR Ca^{2+} content. We suggest that this may form part of an important system whereby changes of SERCA activity produce useful changes of Ca^{2+} release from the SR without the need to have large and energetically costly changes of SR Ca^{2+}.

Methods

All procedures accord to the UK Animals (Scientific Procedures) Act 1986 and University of Manchester Ethical Review Process. Rats (male Wistar, 200–250 g) were killed by stunning and cervical dislocation and ventricular myocytes were isolated as described previously (Eisner *et al.* 1989; Dibb *et al.* 2007). Voltage clamp control was imposed using the perforated patch technique with amphotericin-B (240 μg ml^{-1}). Patch pipettes had a resistance of 2–3 MΩ and contained (in mM): KCH_3O_3S 125, KCl 20, NaCl 10, Hepes 10, $MgCl_2$ 5, K_2EGTA 0.1, titrated to pH 7.2 with KOH. The final access resistance was typically ∼20 MΩ and was overcome using the switch-clamp facility of an Axoclamp-2A amplifier (Axon Instruments). In experiments such as those of Figs 1 and 2, cells were held at a membrane potential of –60 mV and then depolarized with a 10 ms ramp to –40 mV to inactivate the sodium current, followed by a 100 ms pulse to 0 mV at 0.25 Hz. The superfusing solution contained (in mM): NaCl 140, Hepes 10, glucose 10, $CaCl_2$ 1, $MgCl_2$ 1, KCl 4, 4-aminopyridine 5, $BaCl_2$ 0.1, probenecid 2, titrated to pH 7.34 with NaOH. All experiments were performed at 37°C.

Measurement of $[Ca^{2+}]_i$

In the experiments illustrated in Figs 1 and 2 cells were loaded with the Ca^{2+}-sensitive indicator fluo-5F (Molecular Probes) by incubation with the acetoxymethyl (AM) ester (5 μM for 10 min). Fluorescence was converted to $[Ca^{2+}]_i$ using a published method (Trafford *et al.* 1999). Briefly, at the end of the experiment the cell was damaged with the patch pipette. This resulted in an abrupt increase of $[Ca^{2+}]_i$ to levels that saturate the indicator and this was taken as the maximum fluorescence (F_{max}). Assuming that fluorescence is zero in the absence of Ca^{2+} then $[Ca^{2+}]_i$ can be calculated from the level of fluorescence (F) as follows:

$$[Ca^{2+}]_i = K_d F/(F_{max} - F)$$

The K_d was taken to be 1035 nM.

Partial inhibition of SERCA with thapsigargin

Thapsigargin (Sigma-Aldrich, UK) was stored as 1 mM stock solution in DMSO and made up to a final concentration of 1 μM. After obtaining a control recording, the cell was exposed to thapsigargin for between 70 and 130 s and the thapsigargin was then removed. The principle behind this approach is that thapsigargin

is an irreversible inhibitor of SERCA and a fraction of the SERCA molecules will be inhibited permanently. Recordings were then made with SERCA partly inhibited. Thapsigargin was reapplied to further inhibit SERCA and more data obtained. This process was continued for two to four exposures to thapsigargin.

Measurement of SR Ca^{2+} content

SR Ca^{2+} content was measured by applying caffeine (10 mM) and integrating the resulting sodium–calcium exchange (NCX) current (Varro *et al.* 1993). This was then corrected for non-NCX mediated Ca^{2+} efflux as described previously (Díaz *et al.* 1997). In the experiment of Fig. 3 the SR Ca^{2+} content rose to such high levels that the caffeine-evoked rise of $[Ca^{2+}]_i$ resulted in a very large contracture which disrupted the patch. To overcome this we used a modification of a technique published recently (Kashimura *et al.* 2010). The new protocol involves the application of 10 mM 2,3-butanedione monoxime (BDM) to block contraction followed a few seconds later by 20 mM caffeine (in the maintained presence of BDM). BDM has a caffeine-like action and releases Ca^{2+} from the SR (Adams *et al.* 1998) and in order to calculate the SR Ca^{2+} content one has to add the integrals of both the BDM- and caffeine-evoked NCX currents.

Estimation of SERCA activity

k_{SERCA}, the SR-dependent part of the rate constant of decay of the systolic Ca^{2+} transient (Díaz *et al.* 2004) was measured by subtracting the rate constant of decay of the caffeine evoked transient (k_{caff}) from that of the systolic Ca^{2+} transient (k_{sys}). This assumes that the decay of the systolic Ca^{2+} transient is contributed to by a combination of SR and surface membrane whereas the SR does not contribute to the decay of the caffeine response.

Statistics

Data are presented as means ± standard error of the mean of n experiments. Where appropriate, comparisons are made with Student's paired t test ($P < 0.05$).

Results

Figure 1*A* shows the effects of thapsigargin on $[Ca^{2+}]_i$ measured in a rat ventricular myocyte. The cell was initially

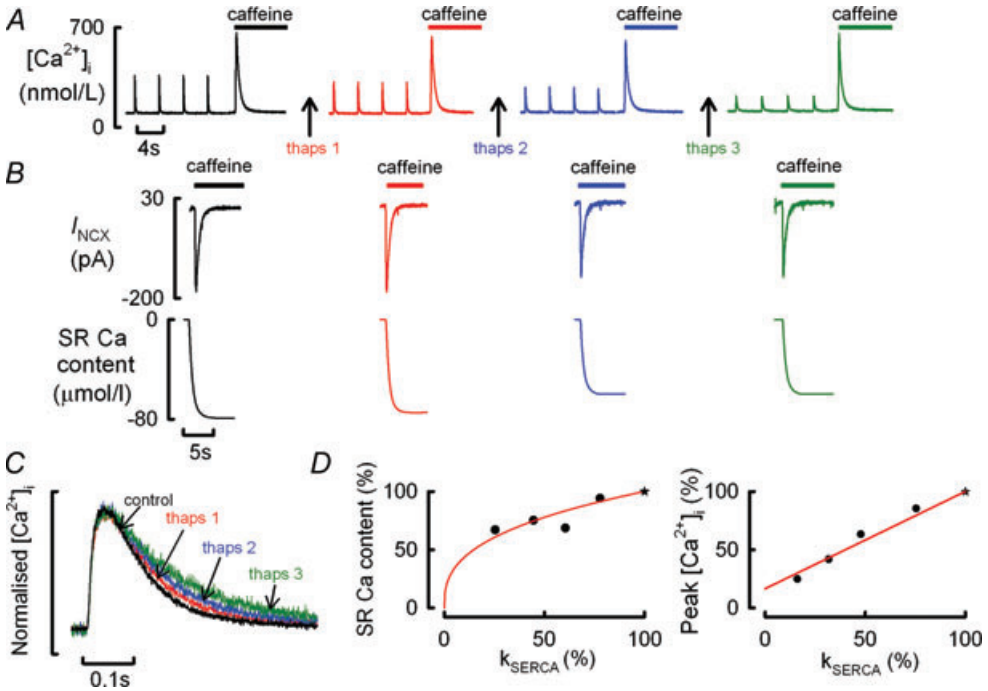

Figure 1. The effects of exposure to thapsigargin on SR Ca^{2+} content
A, time course of experiment. Each trace shows Ca^{2+} transients produced by depolarizing voltage clamp pulses (100 ms duration from −40 to 0 mV at 0.25 Hz). In between such successive recording, thapsigargin (1 μM) was applied for 96, 66 and 113 s, respectively. *B*, estimation of SR Ca^{2+} content. Each record shows the effect of the addition of caffeine (10 mM, filled bar). Traces show: top, membrane current; bottom, integrated current expressed in terms of SR Ca^{2+} content. *C*, normalized systolic Ca^{2+} transients for the period identified. *D*, graphs showing the dependence of SR Ca^{2+} content (left) and peak systolic $[Ca^{2+}]_i$ (right) on k_{SERCA}. The control points are shown by the stars. The curve through the SR Ca^{2+} content data is a fit to SR Ca^{2+} content $= 100 \times (k_{SERCA}/100)^b$, which forces the fit to go through (100,100; star) where $b = 0.36$. The peak systolic $[Ca^{2+}]_i$ data are fitted with a linear regression forced through the control data (100,100; star).

stimulated in the absence of thapsigargin and caffeine (10 mM) was then added to measure SR Ca^{2+} content. The next trace (thaps 1) shows a similar protocol after the cell had been exposed to thapsigargin (1 μM) for 94 s. There is a clear decrease in the amplitude of the systolic Ca^{2+} transients but rather little effect on that of the caffeine-evoked increase of $[Ca^{2+}]_i$. Further decreases in the amplitude of the systolic Ca^{2+} transient were observed following two more exposures to thapsigargin (right hand traces). The quantitative measurements of SR Ca^{2+} content, obtained from the integrated sodium calcium exchange (NCX) currents are shown in Fig. 1*B*. These demonstrate that repeated application of thapsigargin decreases SR Ca^{2+} content. In this example the last exposure to thapsigargin decreased SR Ca^{2+} content to 75% of control. Figure 1*C* illustrates the effects of thapsigargin on the rate of decay of the Ca^{2+} transient. The normalized traces show a clear slowing of the Ca^{2+} transient with exposure to thapsigargin. The SR-dependent rate constant of relaxation (k_{SERCA} – see Methods) in the last record is 32% of that in control. Comparing this with the effect on SR Ca^{2+} content shows that there is a much larger fractional effect on k_{SERCA} than on SR Ca^{2+} content. This is emphasised by the full dataset from this cell (Fig. 1*D*). Figure 1*D* also shows (right hand graph) that, in contrast to SR Ca^{2+} content, the amplitude of the systolic Ca^{2+} transient is a linear function of k_{SERCA}.

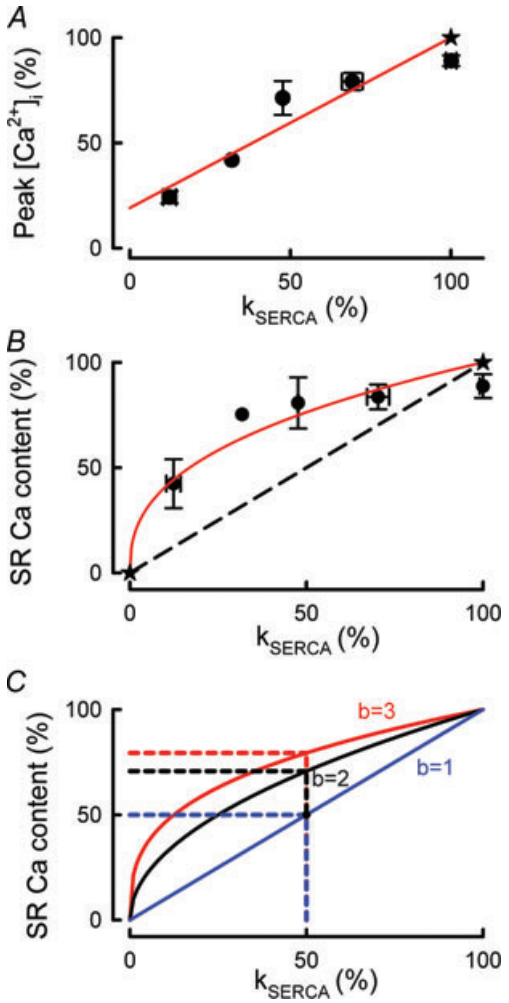

Figure 2. Mean data for the effects of changes of SERCA activity on the amplitude of the systolic Ca^{2+} transient and SR Ca^{2+} content
Data were calculated by measuring k_{SERCA}, peak Ca^{2+} and SR Ca^{2+} content following exposure to thapsigargin. Measurements are normalized to control values in each cell. The data have been grouped into ranges of k_{SERCA} and the means and SEM (from 8 cells) are plotted. Control data are shown as stars. *A*, dependence of peak Ca^{2+} on k_{SERCA}. The line through the data is a linear regression constrained to go through (100,100). *B*, dependence of SR Ca^{2+} content on k_{SERCA}. The continuous red curve is a fit to SR Ca^{2+} content $= 100 \times (k_{SERCA}/100)^b$, which forces the fit to go through (100,100; star). $b = 0.38$. The dashed line is the line of identity. *C*, model for the effects of SERCA activity on SR Ca^{2+} content. The equations are described in the text. The curves show calculations for $b = 1$, 2 and 3. The dashed lines show the predicted SR Ca^{2+} content when SERCA activity has been reduced to 50% of control.

Pooled data from the eight cells studied are shown in Fig. 2. The graph of Fig. 2*A* shows the dependence of the amplitude of the systolic Ca^{2+} transient on k_{SERCA}. The data have been normalized to the control values in the absence of thapsigargin. It is clear that the amplitude is linearly related to SERCA activity. In contrast, as shown in Fig. 2*B*, the dependence of SR Ca^{2+} content on SERCA activity is markedly non-linear. The data can be fitted by a power function such that SR Ca^{2+} content is proportional to $(k_{SERCA})^b$. The curve has been forced through both the control values (shown by the star at (100,100) and the origin (0,0)). The mean value of the exponent, b, was 0.38 ± 0.066. Comparison with the dashed line of identity emphasises that a decrease in SERCA activity (from 100%) results in a much smaller fractional decrease of SR Ca^{2+} content. The left hand point is particularly instructive; a decrease of SERCA activity to 12% of control results in a SR Ca^{2+} content of 42% of control.

Relative role of pump and leak

A previous study has also found that decreasing SERCA activity with thapsigargin produces only a small effect on SR Ca^{2+} content (Ginsburg *et al.* 1998). That study explained the result by suggesting that SERCA operates near thermodynamic equilibrium so that partial inhibition would have little effect on SR Ca^{2+} content. Previous work has shown that inhibiting Ca^{2+} leak from the SR with 100 μM tetracaine increases SR Ca^{2+} content suggesting that SERCA cannot be at equilibrium (Györke *et al.* 1997; Overend *et al.* 1997; Overend *et al.* 1998). The purpose of the final experiments in this paper was to see how far from equilibrium SERCA can operate. The experiment of Fig. 3 shows that adding a high concentration of

tetracaine (1 mM) reversibly elevates SR Ca^{2+} content from 96.8 ± 7.5 to $435.6 \pm 43.2\ \mu mol\ l^{-1}$ ($n = 8$, $P < 0.001$). This confirms SERCA is not operating near equilibrium.

Discussion

The main result in this paper is that a given reduction of SERCA activity results in a quantitatively smaller reduction of SR Ca^{2+} content. These results, produced by acute application of thapsigargin are similar, qualitatively and quantitatively to those found in mice where SERCA activity was decreased by conditional gene knockdown (Andersson *et al.* 2009). It is therefore important to consider the origin of this relationship between SERCA activity and SR Ca^{2+} content.

Model for the relationship between SR Ca^{2+} content and SERCA activity during regular stimulation.

We analyse the experiments with a simple model which assumes that the systolic Ca^{2+} transient is derived from two sources. (1) Ca^{2+} entering the cell via the L-type Ca^{2+} current. We assume that this is constant and ignore the fact that changes in the amplitude of the systolic Ca^{2+} transient affect the rate of inactivation of the Ca^{2+} current and thence the Ca^{2+} entry (Sipido *et al.* 1995; Trafford *et al.* 1997). (2) Ca^{2+} is released from the SR. Previous work (Bassani *et al.* 1995; Trafford *et al.* 1997; Trafford *et al.* 2000) has shown that the Ca^{2+} release from the SR is a steep function of SR Ca^{2+} content ($[Ca^{2+}]_{SR}$) such that $Ca_{rel} = ([Ca^{2+}]_{SR})^b$ where b is of the order of 3. We therefore assume that the amount of Ca^{2+} released from the SR is $m([Ca^{2+}]_{SR})^b$ where m is independent of SR Ca^{2+} content. It should be noted that the exact value of b has no simple physical meaning and will depend, *inter alia*, on the dependence of RyR open probability and single channel current on SR Ca^{2+}.

The amount of Ca^{2+} entering the cell is i_{Ca}. Therefore the increase of cytoplasmic total Ca^{2+} is given by:

$$\Delta Ca = m([Ca]_{SR})^b + i_{Ca} \quad (1)$$

If β is the buffering power defined as the ratio of total divided by free cytoplasmic Ca^{2+} then the amplitude of the systolic *free* Ca^{2+} transient is given by:

$$\Delta[Ca^{2+}]_i = \{m([Ca]_{SR})^b + i_{Ca}\}/\beta \quad (2)$$

We assume that Ca^{2+} uptake into the SR and pumping out of the cell are both linear functions of $[Ca^{2+}]_i$ such that:

$$\text{Ca efflux} = q[Ca^{2+}]_i$$

$$\text{Ca uptake into SR} = p[Ca^{2+}]_i$$

Therefore, a constant fraction, $q/(p+q)$, of the total Ca^{2+} transient is pumped out of the cell. Therefore:

$$\text{Ca efflux} = \frac{q}{(p+q)}\{m([Ca]_{SR})^b + i_{Ca}\} \quad (3)$$

In the steady state Ca^{2+} influx is equal to efflux and therefore:

$$i_{Ca} = \frac{q}{(p+q)}\{m([Ca]_{SR})^b + i_{Ca}\} \quad (4)$$

and it can be shown that:

$$[Ca]_{SR} = p^{1/b}\{i_{Ca}/(mq)\}^{1/b} \quad (5)$$

Thus, SR Ca^{2+} content is predicted to depend on a low $(1/b)$ power of SERCA activity (p).

Substituting for $[Ca^{2+}]_{SR}$ from eqn (5) into eqn (2) gives:

$$\Delta[Ca^{2+}]_i = \{(p/q) + 1\} i_{Ca}/\beta \quad (6)$$

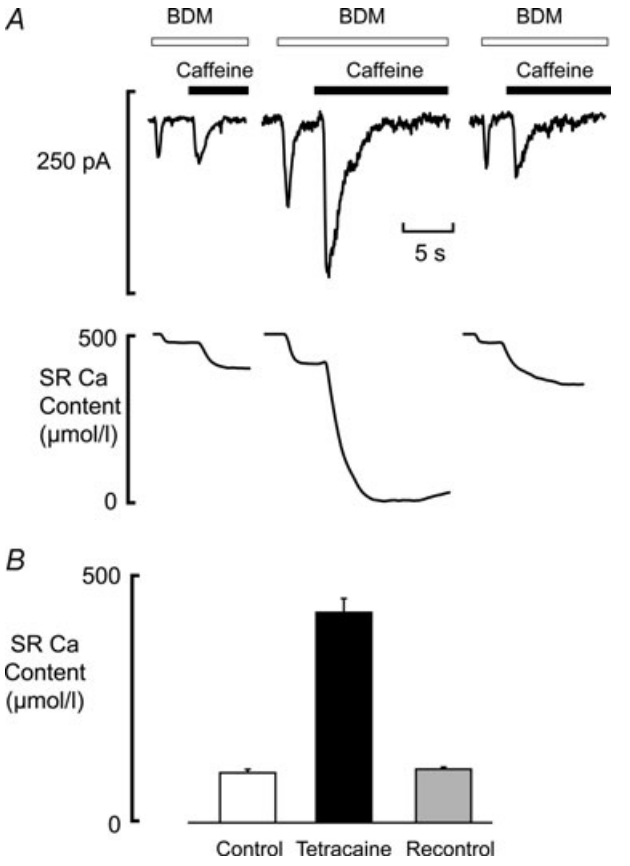

Figure 3. Tetracaine produces a large increase of SR Ca^{2+} content
A, original data. Records show the effect on membrane current (top) and its integral (below) of adding first BDM (10 mM) and then caffeine (20 mM) to release Ca^{2+} from the SR. From left to right traces were obtained in the following conditions: control; after 15 min exposure to tetracaine (1 mM); recontrol. *B*, mean data showing SR Ca^{2+} content in control, tetracaine and recontrol.

In other words, the amplitude of the Ca^{2+} transient, $\Delta[Ca^{2+}]_i$, is a linear function of p and has a value i_{Ca}/β when SERCA is completely inhibited ($p = 0$).

The model therefore predicts that SR Ca^{2+} content will be proportional to the $1/b$ power of SERCA activity. Figure 2*C* shows plots of this relationship for various values of b. Assuming that $b = 3$ then eqn (5) predicts that a 50% reduction of SERCA activity ($p = 0.5$) would result in $[Ca^{2+}]_{SR}$ falling to $0.5^{1/3} = 0.80$ of control. This relationship is summarised in Fig. 2*C*. Consistent with this argument, the mean data of Fig. 2*B* shows that a 75% reduction of SERCA activity with thapsigargin (as measured by the slowing of the decay of the systolic Ca^{2+} transient) only reduces SR Ca^{2+} content by 25% as assessed by the application of 10 mM caffeine. It should be noted that the present model assumes that all the Ca^{2+} efflux from the SR occurs during systole and ignores diastolic leak. We have also produced a model (not shown) which assumes that all the efflux occurs as leak. As long as this leak also depends on SR Ca^{2+} content in a steep manner, the results of the model are identical to those of the present one.

The relative lack of effect of SERCA inhibition on SR Ca^{2+} content has been noted previously. Ginsburg *et al.* (1998) found that decreasing SERCA activity by 39% resulted in a decrease of SR Ca^{2+} content of only 5 to 23%. These authors suggested that the relative lack of change of SR Ca^{2+} arose because SERCA was operating near thermodynamic equilibrium and therefore that decreasing its rate would have little effect on SR content. This is unlikely to be the case in the present paper since, as shown in Fig. 3, decreasing SR Ca^{2+} leak with tetracaine produces a very large increase of SR Ca^{2+} content arguing that SR content is limited by leak of Ca^{2+} from the SR.

Physiological significance

The experimental results show that the amplitude of the systolic Ca^{2+} transient is a linear function of SERCA activity (Fig. 2*A*). This apparently simple relationship, however, results from a combination of two very non-linear relations: (i) SR Ca^{2+} content has a shallow dependence on SERCA activity (Fig. 2*B*) and (ii) as previously shown Ca^{2+} release from the SR depends steeply on SR content (Bassani *et al.* 1995; Trafford *et al.* 1997, 2000). This paper has characterised these relations. The final point concerns what, if any, is the physiological significance of these non-linear curves. On this point one can only speculate that the observed relationships mean that stimulation of SERCA will produce a positive lusitropic and inotropic effect without a correspondingly large increase of SR Ca^{2+} content. Given that increasing SR Ca^{2+} content is energetically costly this may be a useful adaptation.

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Author contributions

D.A.E., S.C.O. and A.W.T. designed the experiments and wrote the paper. E.F.B., S.J.B. and C.L.O. performed the experiments and analysed the data. All authors approved the final version of the paper. Experiments were performed at the University of Manchester apart from some preliminary studies at the University of Liverpool.

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