Isolated Rabbit Working Heart Function During Progressive Inhibition of Myocardial SERCA Activity

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Rationale: The extent to which sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) activity alone determines left ventricular (LV) pump function is unknown.

Objective: To correlate SERCA activity with hemodynamic function of rabbit LV during thapsigargin perfusion.

Methods and Results: Isolated rabbit hearts were perfused in working heart configuration, and LV pump function was assessed using a pressure-volume catheter. Rapid and complete (>95%) inhibition of SERCA was associated with a moderate decrease in cardiac function (to 70%–85% of control). Further decrease in cardiac function to 50%–75% of control occurred over the next ≈30 minutes despite no detectable further inhibition of SERCA activity. Analysis of the 20 seconds prior to pump failure revealed a rapid decrease in end diastolic volume. Intermediate levels of SERCA function (∼50% of control) had only minor hemodynamic effects. Parallel experiments in field-stimulated isolated ventricular cardiomyocytes monitored intracellular Ca\(^{2+}\) and cell shortening. On perfusion with thapsigargin, Ca\(^{2+}\) transient amplitude and cell shortening fell to ∼70% of control followed by increased diastolic Ca\(^{2+}\) concentration and diastolic cell shortening to achieve a new steady state.

Conclusions: The relationship between SERCA activity and LV function in the rabbit is highly nonlinear. In the short term, only moderate effects on LV pump function were observed despite almost complete (>95%) reduction in SERCA activity. The terminal decline of function was associated with sudden sustained increase in diastolic tone comparable to the sustained contraction observed in isolated cardiomyocytes. Secondary increases of intracellular Ca\(^{2+}\) and Na\(^{+}\) following complete SERCA inhibition eventually limit contractile function and precipitate LV pump failure. (Circ Res. 2012;110:1618-1627.)

Key Words: sarcoplasmic reticulum Ca\(^{2+}\) ATPase ■ intracellular calcium ■ myocardial contraction

Ventricular cardiomyocytes isolated from animal models of cardiac disease and patients with heart failure demonstrate altered expression and function of a number of Ca\(^{2+}\) handling proteins important for excitation-contraction coupling (E-C coupling).\(^{1-4}\) One such protein is the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase pump (SERCA), which plays a pivotal role in both myocardial contraction and relaxation. SERCA functions to pump Ca\(^{2+}\) into the sarcoplasmic reticulum (SR) store. In doing so, it returns the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) to resting levels causing relaxation of the cardiomyocyte and replenishment of the SR Ca\(^{2+}\) store for the next cycle.

To fully understand the impact of SERCA function on the physiology and patho-physiology of cardiac muscle, it is important to quantify the specific contribution of SERCA to whole heart pump function. Transgenic methodology, by allowing selective removal/reduction of one protein, has been used to examine the relationship between cardiac SERCA levels and whole heart function in mice.\(^{5-8}\) However, these chronic reductions of SERCA protein cause compensatory changes in expression of other proteins (eg, L-type Ca\(^{2+}\) channel, sodium/calcium exchanger [NCX], phospholamban) altering the status of E-C coupling and changes in whole heart structure and myofilament sensitivity, all of which prevent a direct assessment of the role of SERCA in isolation. Furthermore, the electrophysiology and E-C coupling process in rodent hearts is distinct in many aspects from that of larger mammals, in particular humans.\(^{9,10}\)

The major aim of this study was to quantitatively assess the impact of SERCA activity alone on whole heart mechanical function. We hypothesize that in the complete absence of SR...
activity (<5%), left ventricular (LV) contractility is insufficient to maintain normal pump function. Acute pharmacological blockade of SERCA activity was achieved using the selective inhibitor thapsigargin (TG) in a rabbit isolated working heart preparation combined with pressure-volume catheter technology to monitor whole heart contractile and hemodynamic function.

The novelty and advantage of this experimental approach is several fold: (1) it uses a species with E-C coupling more similar to that of human, (2) acute inhibition negates the compensatory alterations of transgenics, and (3) there are no changes in vascular resistance (ie, changes in pre/afterloads or centrally-mediated autonomic signaling). Whole heart experiments were performed under near physiological conditions and parallel studies with isolated rabbit cardiomyocytes investigated the cellular mechanisms underlying the response to acute SERCA inhibition with TG. The data demonstrated that SERCA activity was rapidly reduced to below 5% in the first 4 to 5 minutes of perfusion with TG yet working heart function was maintained for up to 60 minutes. Pump function eventually failed due to a sustained increase in diastolic tone with preserved ejection fraction in the absence of arrhythmic events.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Working Heart Set-Up and LV Pressure-Volume Measurement

Hearts were excised from adult male New Zealand White rabbits and cannulated onto an isolated working heart perfusion system via the aorta and pulmonary vein of the left atrium. Perfusion was set in working heart mode (Online Figure I); preload and afterload were set at 6 mm Hg and 60 mm Hg respectively. Aortic flow (AoF) was measured continuously by an in-line ultrasonic flow-meter (Malema) and hearts were paced at 211±5 bpm. A custom-built 3F variable segment pressure-volume catheter (Scisense, Canada) was advanced past the aortic valve into the LV for continuous measurement of hemodynamic function. The catheter allowed selection of volume segments of 11.0, 14.0, 17.0, or 19.0 mm. Parallel conductance was estimated by injection of a bolus of hypertonic saline into the preload chamber of the isolated working heart system.11

Experimental Protocol

Hearts were assigned to 1 of 2 perfusion protocols as shown in Figure 1. Corresponding vehicle control groups (Protocol 1, n=5; Protocol 2, n=7) were produced by perfusion with an equivalent volume of DMSO for time-matched durations.

Protocol 1 (>5 Minutes)

Hearts were perfused with 3 μmol/L TG (n=6) and LV function was continuously recorded until AoF was undetectable (defined as <20 mL/minute on flowmeter; Online Figure II). Hearts were subsequently switched and perfused in nonworking heart mode with (1) nominally Ca2+-free Tyrode’s solution containing 50 μmol/L ethylene glycol tetraacetic acid (EGTA) (2 minutes) followed by (2) 3 μmol/L TG (0.5 minutes). These final 2 perfusion steps served to buffer the circulating free [Ca2+] in the tissue to 100 to 200 Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AoF</td>
<td>aortic flow</td>
</tr>
<tr>
<td>[Ca2+]i</td>
<td>intracellular Ca2+ concentration</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>[Na+]i</td>
<td>intracellular Na+ concentration</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium/calcium exchanger</td>
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<tr>
<td>PP</td>
<td>peak pressure</td>
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<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticulum Ca2+ ATPase</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>SW</td>
<td>stroke work</td>
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Figure 1. Isolated working rabbit heart protocols. Hearts subject to Protocol 1 were perfused with 3 μmol/L thapsigargin (TG) until aortic flow (AoF) fell to undetectable levels. Hearts subject to Protocol 2 were perfused with 3 μmol/L TG for short time periods until DP/dtmax dropped by between 5%–22% of control. Time ranges of TG perfusion for each group are shown; time-matched vehicle controls were obtained by perfusion with DMSO. Following the TG perfusion period, both groups were perfused in nonworking heart mode (retrograde perfusion) with ethylene glycol tetraacetic acid (EGTA) (2 minutes) followed by (2) 3 μmol/L TG (0.5 minutes). These final 2 perfusion steps served to buffer the circulating free [Ca2+] in the tissue to ~100 to 200
mmol/L (required for the in vitro SERCA activity assay) and to act as a control in the vehicle group for any residual circulating TG in the vasculature at the end of the experiment respectively.

Protocol 2 (<5 Minutes)
For correlation of working heart contractile function with SR Ca
2+
uptake, hearts were perfused with 3 μmol/L TG (n = 10) for short periods of time (1–4 minutes) and LV function was continuously recorded until dP/dt max dropped by 5% to 22% of control values.

Tissue Homogenization
Immediately at the end of each working heart perfusion protocol, a transmural section of LV free wall was cut and snap frozen in liquid nitrogen. Tissue was homogenized in five volumes of ice cold protease/phosphatase inhibitor buffer and stored at ~80°C.

SR Ca
2+
Uptake Studies
Total protein content of the whole LV homogenate was assessed using the Bradford protein assay. Oxalate supported SR Ca
2+
uptake measurements were performed as described previously
12
and converted to [Ca
2+
]i as detailed in the Online Supplement. SERCA activity was quantified in the hearts with measurable uptake by fitting gradients to the decline in free [Ca
2+
]i over a 50 mmol/L range at 0.3, 0.5 and 1 μmol/L and expressed relative to those from the time-matched vehicle hearts.

Field Stimulation of Intact Cardiomyocytes With Simultaneous Whole-Cell Epifluorescence and Shortening Measurements
Studies were performed on rabbit LV cardiomyocytes as described previously.
3,14
Intact cardiomyocytes were loaded with Fura-2AM (5 μmol/L; Invitrogen) and placed in a bath on an inverted microscope. To parallel working heart conditions, cells were perfused at 37°C with modified Krebs-Henseleit with 2.5 mmol/L [Ca
2+
]i and field stimulated at ~3.5 Hz. Cells were then perfused with 3 μmol/L TG for variable durations (5–240 seconds) prior to rapid application of 15 mmol/L caffeine. The Fura-2 fluorescence (340 and 380 nm excitation; R340/380 nm) was measured using a spinning wheel spectrophotometer (Cairn Research, UK; sampling rate of 500 Hz) whereas cellular shortening was measured using a video edge detection system (IonOptix, MA; sampling rate of 200 Hz).

Data Analysis and Statistics
All data were analyzed offline and expressed as mean±SEM.

For isolated working heart studies, mean pressure and volume signals were obtained by averaging (1) 10 cardiac cycles in control and 5 minutes post-TG (TG+5 minutes) in Protocol 1 and (2) the final 4 cardiac cycles in the presence of TG at the end of each protocol.

For isolated cardiomyocyte studies, mean [Ca
2+
]i and cell shortening signals were obtained by averaging 6 transients (OriginPro v6.1). In cases of multiple comparisons either repeated-measures ANOVA with Tukey-Kramer post test correction (working heart function and uptake gradient data) or 1-way ANOVA with Dunnett post test correction (working heart function during the final 60 seconds and Ca
2+
 transient/cell shortening parameters at sequential time points in TG) were used (GraphPad InStat). Values were considered significant when P<0.05.

Results
Effects of Acute SERCA Inhibition on Whole Heart Working Rabbit Heart Function: Protocol 1
The effects of TG perfusion and time-matched vehicle perfusion during Protocol 1 on mechanical function of the working rabbit heart are summarized in Figure 2. Control working heart function was not significantly different between groups prior to treatment with either TG or vehicle solution. Figure 2A(i) shows typical LV pressure (top), volume (middle), and AoF (bottom) recordings from an isolated working heart perfused with 3 μmol/L TG. Panels from left to right depict control, 5 minutes postaddition of TG (TG+5 minutes) and at the point where AoF fell below detectable levels (< 20 mL/minute, end point, denoted by the dashed box). Addition of TG led to an initial rapid decline in LV function over the first 5 minutes of perfusion, after which a pseudo steady state was achieved and maintained for variable periods of time (maximum ~60 minutes). This was followed by a rapid decline in function before AoF fell to undetectable levels. The mean time for AoF to reach undetectable levels following the TG perfusion in Protocol 1 was 36.3 ± 10.2 minutes. Figure 2A(ii) shows the pressure-volume loops corresponding to the recordings in Figure 2A(i). As perfusion with TG progressed there was a leftward shift of the pressure-volume loop accompanied by a decline in stroke volume. These alterations in volume parameters are summarized in Figure 2A(iii). Consequently, despite the decline in stroke volume following TG perfusion, ejection fraction (EF; Figure 2A(iv)) was not significantly altered between the 3 points (Control: 59.5 ± 2.5%, TG+5 minutes: 54.3 ± 4.6% and end point: 65.9 ± 6.0%; P>0.05). Coronary flow was unaltered throughout the protocol (Figure 2A[v]); Control: 84.6 ± 2.0, TG +5 minutes: 84.9 ± 1.8 and end point: 82.3 ± 1.0 mL/min; P>0.05).

Figure 2B(i–iii) shows indices of contractile function at 5 minutes postaddition (TG+5 minutes) and at end point in TG and vehicle-treated hearts. Perfusion with TG resulted in a significant decline in all contractile parameters at 5 minutes postaddition and at end point. Peak pressure (PP), dP/dt max, and stroke work (SW) were all significantly reduced 5 minutes postaddition of TG (PP: 108.1 ± 2.3 versus 93.8 ± 2.0 mm Hg; dP/dt max: 2031 ± 175 versus 1370 ± 48 mm Hg/s; SW: 86.1 ± 4.5 versus 51.8 ± 2.7 mm Hg*mL; control versus TG+5 minutes for all parameters; P<0.05). This decline continued to the end point at which PP had fallen to 80.4 ± 2.2 mm Hg, dP/dt max to 1010 ± 18 mm Hg/s and SW to 22.5 ± 2.5 mm Hg*mL (P<0.05 control versus end point for all parameters). In vehicle hearts at end point, there was a small but significant decline in dP/dt max and SW (dP/dt max: 2152 ± 141 versus 1916 ± 128 mm Hg/s; SW: 68.5 ± 9.9 versus 59.8 ± 9.4 mm Hg*mL; control versus end point; P<0.05).

Working heart diastolic indices are shown in Figure 2C(i–iii). TG perfusion led to significant increases in end diastolic pressure (Figure 2C(i)) and the relaxation constant Tau (Figure 2C(ii)) at 5 minutes postaddition and at the end point of the experiment (end diastolic pressure: 7.7 ± 1.5 versus 12.4 ± 1.1 versus 13.4 ± 1.3 mm Hg; Tau: 27.0 ± 3.1 versus 40.7 ± 6.0 versus 69.8 ± 8.8 ms; SS versus TG+5 minutes versus end point, P<0.05). In addition, TG perfusion led to a significant depression in dP/dt min after 5 minutes which was more pronounced at end point (SS: -2347 ± 143 versus TG +5 minutes: 1712 ± 134 versus end point: -1341 ± 120 mm Hg/s; P<0.05; Figure 2C(ii)). There were no significant changes in diastolic indices in the vehicle group.
The example trace of AoF for the complete time course in TG (Figure 3A) demonstrates the rapid drop in LV function, which immediately preceded pump failure in Protocol 1 TG treated hearts. Closer inspection of the 60 seconds prior to cessation of AoF dissected the changes in functional parameters during this phase with improved resolution (Figure 3B). AoF was significantly reduced at 20 and 10 seconds prior to end point and thereafter fell precipitously toward undetectable levels. Over the course of the final minute, pressure indices remained unchanged until end point at which PP, dP/dtmax, and dP/dtmin were significantly reduced ($P<0.05$ versus −60-second timepoint). End diastolic volume, end systolic volume, and SW were significantly reduced at −10 seconds and end point ($P<0.05$ versus −60-second timepoint) thus confirming the rapid time course of the cessation of working heart function in TG-treated hearts where changes in volume appeared to precede changes in pressure.

Figure 2. Protocol 1: The influence of progressive sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) inhibition on working rabbit heart function. A(i), Representative left ventricular pressure (LVP), volume, and aortic flow (AoF) traces under control conditions (pre-thapsigargin [TG]; left panel), 5 minutes after perfusion of TG (middle panel) and at end point (undetectable AoF; right panel); A(ii), Corresponding pressure-volume loops at each time point; A(iii), Absolute volume measurements from TG perfused hearts at indicated time points (end diastolic volume [EDV], light gray bars; end systolic volume [ESV], dark gray bars; n = 3; #P < 0.05 vs control; $\psi P < 0.05$ vs TG + 5 minutes); A(iv), calculated ejection fraction at each time point; and A(v), calculated coronary flow at each time point. B(i–iii), Mean indices of contractile function, expressed as % of control, 5 minutes after drug addition, and at end point in vehicle (white bars; n = 5) and TG (gray bars, n = 6) hearts. PP indicates peak pressure; SW, stroke work. C(i–iii), Mean indices of diastolic function expressed as in B. *$P < 0.05$ vs control; $\# P < 0.05$ TG + 5 minutes vs end point. End point data were consistently taken from the final 4 cardiac cycles following cessation of detectable AoF (dashed box A(i), right panel).
Figure 3. Rapid timecourse of cessation of working heart function in response to Protocol 1. A. Complete time course of aortic flow (AoF) in a heart subject to thapsigargin (TG) perfusion in Protocol 1 showing the precipitous decline in flow in the 60 s that preceded end point (dashed box). B. Expanded plots of mean pressure (top) and volume (bottom) indices in the final 60 s that preceded undetectable AoF (AoF is shown on both; black squares). Data are expressed as a percentage of the −60 s timepoint (baseline). AoF was significantly reduced at 20 and 10 s time points before falling to undetectable levels. Pressure parameters were not significantly reduced until end point (n=6; P<0.05 with exception of end diastolic pressure [EDP]) although all volume parameters were significantly reduced earlier, and to a greater degree than that of pressure, at the −10 s timepoint and end point (P<0.05; n=3). PP indicates peak pressure; EDV, end diastolic volume; ESV, end systolic volume; SW, stroke work; CF, coronary flow.

SERCA Activity in Working Hearts Treated With TG

In order to determine SERCA activity at the end of working heart experiments subject to either Protocol 1 (>5 minutes) or Protocol 2 (<5 minutes), a section of LV free wall was immediately taken from the whole heart, snap frozen, and homogenized. Homogenates (4 mg/mL total protein) were then subject to a cuvette-based oxalate-facilitated Ca^{2+} uptake assay. Vehicle-treated hearts subject to either protocol consistently demonstrated a rapid Ca^{2+} uptake component (mean maximum uptake rate and dissociation constant were 2.96±0.25 nmole/mg total protein/minute and 243±16.3 nmoles/L, respectively, which are within the range of previously published values15–17; Online Figure III). Assay sensitivity was verified (Online Figure IV) and the potential for unbound TG contamination and regional variation were controlled for (Online Figure V & VI).

SERCA Activity in Working Hearts Treated With TG: Protocol 1

All vehicle-treated hearts demonstrated rapid Ca^{2+} uptake as depicted by the decline in free [Ca^{2+}], in the example control traces in Figure 4A (idii, black traces), which became faster in time course with increasing total protein concentration in the cuvette (data not shown). In contrast however, there was no detectable Ca^{2+} uptake from any Protocol 1 TG-treated samples (Figure 4A, gray trace).

SERCA Activity in Working Hearts Treated With TG: Protocol 2

Ten hearts were subject to Protocol 2 and perfused with TG for between 1 to 4 minutes; vehicle hearts were perfused with DMSO and time-matched (n=7). Assessment of SERCA activity revealed 5 of these TG-treated hearts had a measurable uptake component (Figure 4A[ii], gray trace) and 5 hearts did not. SERCA activity was quantified in hearts with measurable uptake as shown in Figure 4A(ii) inset. Comparison of the gradients of decline between these 3 points in each heart showed no significant differences in any of the 5 hearts indicating no alteration in dissociation constant of SERCA following exposure to TG.
Correlation of function with the degree of SERCA activity was performed by examining a range of functional parameters immediately prior to the end of the experiment in TG-treated hearts with and without measurable Ca\(^{2+}\) uptake (Figure 4B). Of these parameters, mean PP, developed pressure, and dP/dt\(_{\text{max}}\) were significantly different between the 2 groups (uptake versus no uptake; PP: 97.6±0.6 versus 94.6±1.0, P<0.05; developed pressure: 97.1±1.0 versus 92.0±2.0, P<0.05; dP/dt\(_{\text{max}}\): 91.0±1.5 versus 83.3±1.34, P<0.001; all % control). The most sensitive functional parameter to TG was dP/dt\(_{\text{max}}\), which showed no overlap in function between hearts with measurable uptake (circles) and those with undetectable uptake (squares; Figure 4B). Linear regression analysis of a plot of individual dP/dt\(_{\text{max}}\) values against SERCA activity (expressed relative to vehicle; Figure 4C) revealed that SERCA activity fell below undetectable levels once dP/dt\(_{\text{max}}\) had fallen below 84% of control function.

Characterization of Ca\(^{2+}\) Transients, Cell Shortening, and SR Ca\(^{2+}\) Content in Single Rabbit Cardiomyocytes Perfused With TG

To assess the effects of TG on [Ca\(^{2+}\)]\(i\), handling and cell shortening under equivalent conditions to working heart experiments (2.5 mmol/L [Ca\(^{2+}\)]\(i\), 5.3 Hz, 37°C), isolated rabbit cardiomyocytes were field stimulated and perfused with TG for varying durations. At the end of TG perfusion, rapid application of caffeine was used to assess SR Ca\(^{2+}\) content. Figure 5A shows typical sections of trace obtained in the solutions and at time points indicated above. The cell in Figure 5A(i) was exposed to TG for 40 seconds, which was followed by a significant response to the subsequent caffeine bolus, whereas the cell in Figure 5A(ii) was exposed to TG for 180 seconds, which resulted in no response to caffeine. TG perfusion times were varied between 5 to 240 seconds before caffeine application (n=12 cells from 6 hearts). A subset of cells was perfused with a comparable percentage of DMSO (0.09%) for time-matched durations to obtain vehicle SR contents. Analysis of the mean peak and diastolic [Ca\(^{2+}\)]\(i\), (Figure 5B[i]) and mean cell shortening (Figure 5B[ii]) transients at set time points during perfusion with TG was performed as described in methods. [Ca\(^{2+}\)]\(i\) transient peak dropped sharply within the first 40 seconds of TG perfusion before slowly increasing thereafter until the 240-second time point, whereas Ca\(^{2+}\) transient diastolic levels continued to steadily rise throughout exposure to TG (Figure 5B[i]). Diastolic [Ca\(^{2+}\)]\(i\), was significantly increased at 220 and 240 seconds post-TG (P<0.05 versus control pre-TG). A comparable pattern of change was seen in peak and diastolic cell shortening during perfusion with TG. The amplitude of the caffeine-induced Ca\(^{2+}\) transient was taken as a measure of SR Ca\(^{2+}\) content both in free [Ca\(^{2+}\)]\(i\), and also converted to total [Ca\(^{2+}\)]\(i\), using previously published cytoplasmic buffer characteristics. All cells perfused with TG for less than 150 seconds (n=6) demonstrated SR Ca\(^{2+}\) release on caffeine application; all those perfused for 150 seconds or longer had no response to caffeine (n=6). A plot of total SR Ca\(^{2+}\) content against TG perfusion time revealed that SR Ca\(^{2+}\) content declined exponentially with increasing exposure time to TG (Figure 5C[i]). As can be seen in Figure 5C(ii) there was a hyperbolic relationship (exponent=3.6) between the mean amplitude of the Ca\(^{2+}\) transient immediately before caffeine application and the total SR Ca\(^{2+}\) content. This relationship suggested that total SR Ca\(^{2+}\) content is reduced to zero when Ca\(^{2+}\) amplitude falls to 52% of control. A similar relationship was demonstrated for cell shortening (data not shown). The decay of the Ca\(^{2+}\) transient is predominantly due to SERCA, accounting for ~70% of Ca\(^{2+}\) removal in the rabbit. Therefore a measure of the contribution of SERCA to the decay of the Ca\(^{2+}\) transient was taken using the gradient of decline of the total [Ca\(^{2+}\)]\(i\), transient over a 10 μmol/L range expressed relative to the control value (Figure 5C[iii]). This plot was fit with an exponential decay, which suggested that the SERCA activity decayed rapidly within the first 50 seconds of TG perfusion reaching a plateau at approximately 60 seconds post-TG.

The averaged free [Ca\(^{2+}\)]\(i\), transients in control, 70 seconds post-TG and 240 seconds post-TG, shown in Figure 5D(i–iii), demonstrate the propensity for a rightwards shift in the peak with increasing durations of TG treatment. Mean time to rise and time to fall were calculated as an indicator of the alteration in Ca\(^{2+}\) transient kinetics to TG under the conditions used in working hearts (Figure 5D[iv]). Both the time to rise and time to fall were significantly altered at the 70-second time point in TG (rise time increased to 185.7±18.3%, whereas fall time decreased to 87.4±2.3% both of control; P<0.05 control versus 70 seconds, n=10 at 70 second time point). At these high (physiological) stimulation rates, TG had no significant effect on the half time of decay of the Ca\(^{2+}\) transient.

Discussion

The main objective of this study was to assess the impact of SERCA activity on cardiac mechanical function in the absence of changes in pre- or afterload on the heart or any major compensatory changes that can occur over the longer term following SERCA downregulation. A secondary consideration was to use a heart where the relative contribution of the SR Ca\(^{2+}\) release to E-C coupling was similar to that of the human heart. These aims were achieved by applying a specific inhibitor of SERCA (3 μmol/L TG) to a rabbit working heart preparation. Initially, SERCA inhibition led to a relatively rapid (within the first 5 minutes) but modest decrease in cardiac function (to ~70%–85% of pre-TG levels) across a range of functional parameters (PP, dP/dt\(_{\text{max}}\), dP/dt\(_{\text{min}}\)). A further decrease in cardiac function to 50% to 75% of pre-TG levels occurred over the next ~30 minutes despite no detectable SERCA activity in these hearts. Parallel experiments of [Ca\(^{2+}\)]\(i\), and cell shortening in isolated rabbit cells suggest that a substantial Ca\(^{2+}\) transient remained after acute SR inhibition accompanied by increased diastolic Ca\(^{2+}\) concentration and diastolic cell shortening. The data suggest that the mechanical deficit associated with an acute yet substantial reduction in SERCA activity (>95%) is insufficient to prevent cardiac pump function directly.
Cardiac Pump Function After Substantial Reductions of SERCA Activity

Cardiac pump function was measured using a pressure-volume catheter in combination with the rabbit working heart. Use of these devices ensured sensitive measurement of various systolic and diastolic functional parameters, which were then correlated with SERCA activity measurements on the same heart. This is in contrast to previous studies where TG and other SERCA inhibitors have been used on isovolumetric ex vivo heart preparations and where vascular effects of TG have been demonstrated. Since coronary flow was unaltered during the protocol in our preparation, the direct effect of reduced SERCA activity on working heart mechanical function could be assessed. When comparing the current working heart model to previous studies on transgenic mice, the relationship between cardiac contractility and SERCA activity shows a similar nonlinear relationship, ie, in the transgenic studies, SERCA reductions of up to 50% have small effects on function, whereas reductions of SERCA of up to 90% have significant effects on contractility yet mechanical pump function can be maintained. In the current working heart model, despite having no detectable SERCA

Figure 5. Effect of progressive sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) inhibition on isolated rabbit cardiomyocyte Ca\(^{2+}\) transient, cell shortening and sarcoplasmic reticulum (SR) Ca\(^{2+}\) content. A, Representative traces of free [Ca\(^{2+}\)]\(_i\) and cell shortening in field stimulated isolated cardiomyocytes perfused for (i) 40 s and (ii) 180 s in 3 \(\mu\)mol/L thapsigargin (TG) followed by rapid application of 15 mmol/L caffeine. B, Mean±SEM values for peak and diastolic (i) free [Ca\(^{2+}\)]\(_i\) and (ii) shortening in cells perfused with 3 \(\mu\)mol/L TG for varying durations; open symbols represent mean control (pre-TG) values (10 s: n=12; 40 s: n=11; 70 s: n=10; 100 s: n=9; 130 s: n=7; 160 s: n=6; 190 s: n=5; 220 s: n=5). Diastolic [Ca\(^{2+}\)]\(_i\), was significantly increased at both 210 and 240 s in TG (P<0.05 vs control pre-TG). C, Relationship of Ca\(^{2+}\) transient amplitude, total SR Ca\(^{2+}\) content, and exposure time to TG; (i), total SR Ca\(^{2+}\) content decreases exponentially with exposure time to thapsigargin; (ii), plot of Ca\(^{2+}\) transient amplitude and total SR Ca\(^{2+}\) content exposed to thapsigargin for varying durations demonstrates a hyperbolic relationship and (iii), SERCA activity (as assessed by the rate of decay of the Ca\(^{2+}\) transient over a 10 \(\mu\)mol/L range expressed relative to the Control Pre-TG value) declines rapidly to a plateau within the first 70 s of TG perfusion. Superimposed open symbols indicate mean±SEM values for SR Ca\(^{2+}\) content in time-matched vehicle cells. D, Averaged Ca\(^{2+}\) transients recorded at ~3.5 Hz in (i) control (pre-TG), (ii) 70 s, and (iii) 240 s post-TG perfusion; (iv) assessment of the mean time to rise (light gray bars) and fall (dark gray bars) revealed significant alterations vs control from 70 s post-TG onwards (P<0.05, all data are expressed as % of control pre-TG; 10 s: n=12; 40 s: n=11; 70 s: n=10; 100 s: n=9; 130 s: n=7; 160 s: n=6; 190 s: n=5; 220 s: n=5; 240 s: n=5).
activity or compensatory changes in protein expression, the
decrease in mechanical function over the first 5 minutes was
also initially limited with a 30% decrease in dP/dt<sub>max</sub> 28%
decrease in dP/dt<sub>min</sub>, and a 13% decrease in PP (Figure 2).
Thereafter, cardiac function in the working heart model
slowly diminished over the next ~30 minutes by an addi-
tional 18% (dP/dt<sub>max</sub>), 15% (dP/dt<sub>min</sub>), and 12% (PP), at
which point the heart failed to produce sufficient aortic flow.
Although the current study examined the effects of SERCA
inhibition alone to whole heart function under near physio-
logical conditions the response under conditions of varying
rates and loads requires future examination.

Effect of Progressive Thapsigargin Exposure on
Isolated Cardiomyocytes

Experiments on single cells from the rabbit at stimulus rates
comparable to working heart experiments showed that the Ca<sup>2+</sup>
transient amplitude and the associated cell shortening was
reduced to ~60% of control after ~60 seconds in the TG
solution (Figure 5B[i&ii]). The rate of Ca<sup>2+</sup> transient decay
was reduced to a steady level after a similar time scale (Figure
5C[iii]), yet caffeine induced Ca<sup>2+</sup> release indicated that
~40% of the SR content remained (Figure 5C[i]). Continued perfusion with TG resulted in the loss of Ca<sup>2+</sup> from the SR
without any significant change in the Ca<sup>2+</sup> transient char-
acteristics. This suggests that the SR contribution to E-C
coupling was not present when the SR Ca<sup>2+</sup> content fell
below ~40% of control; this is consistent with previous measurements. The remaining SR Ca<sup>2+</sup> content slowly
decayed to undetectable levels in the subsequent ~90 seconds (Figure 5C[i]) thus showing a similar time-course to SR
depletion as previously reported for rabbit cells perfused with
TG. Ploting the range of values of SR total Ca<sup>2+</sup> content
and Ca<sup>2+</sup> transient amplitude against the Ca<sup>2+</sup> transient
amplitude suggested a hyperbolic relationship between SR
content and the SR contribution to the Ca<sup>2+</sup> transient char-
acteristics. This steep relationship has been observed by other groups and predicts that when the SR Ca<sup>2+</sup> content decreases to ~50% of control values, the SR component of the Ca<sup>2+</sup> transient will be only ~8% of normal. In rabbit myocardium, the remaining Ca<sup>2+</sup> transient (~50% of control values) resulted from the
large influx via L-type Ca<sup>2+</sup> channel. The involvement of the SR in E-C coupling appeared to be negligible after ~60
seconds in TG, yet diastolic [Ca<sup>2+</sup>] and diastolic cell short-
ening continued to change over the subsequent 200 s. The
reasons for these progressive changes in diastolic [Ca<sup>2+</sup>] are
unclear; one possibility is that the slow rise of diastolic
[Ca<sup>2+</sup>] is caused by a slow increase in intracellular Na<sup>+</sup>
concentration ([Na<sup>+</sup>]i) observed during SERCA inhibition,20
this would raise diastolic [Ca<sup>2+</sup>]<sub>i</sub> via NCX. Additional exper-
iments on isolated single cells confirmed that following
addition of TG, [Na<sup>+</sup>]i increased significantly (by ~3.4 mmol/L, Online Figure VII). This increase is compa-
rable to the measurements made by Maier et al (1997) in
whole hearts.20 On cessation of stimulation, [Na<sup>+</sup>]i fell
rapidly to below pre-TG values (12.0±1.0 versus
5.7±2.8 mmol/L, TG +240 seconds, ~3.5Hz versus TG
+340 seconds, 0Hz, P<0.05) as would be expected due to sarcolemmal extrusion processes.

Changes in Ca<sup>2+</sup> transient kinetics were also observed, the
most pronounced effect being the increase in the time to peak
of the Ca<sup>2+</sup> transient to 185% of control after ~60 seconds
perfusion with TG (Figure 5D[iiv]). This time-course is consis-
tent with the loss of rapid SR Ca<sup>2+</sup> release and the loss of
associated rapid SERCA-mediated reuptake. In the ab-
sence of SR activity, intracellular [Ca<sup>2+</sup>]<sub>i</sub> would continue to
increase even after the L-type Ca<sup>2+</sup> channel current had
decayed due to Ca<sup>2+</sup> entry via NCX during the plateau phase
of the action potential. Repolarization of the action potential
would drive NCX into the forward mode (Ca<sup>2+</sup> extrusion)
abruptly reducing [Ca<sup>2+</sup>]<sub>i</sub>. Thus, as reported previously, time
to peak [Ca<sup>2+</sup>]<sub>i</sub>, in the absence of SERCA activity is deter-
mined by duration of the action potential.27

Causes of Failure of Pump Function in TG

All working heart preparations subject to Protocol 1 eventu-
ally failed to produce an aortic flow as a result of sustained
perfusion with TG. Arrhythmias were not seen at this end
point, instead sinus rhythm was maintained and pump func-
tion fell precipitously over a period of the final 60 seconds
(Figure 3A, dashed box). SERCA activity was undetectable
in preparations with impaired but significant working heart
function and in tissue immediately after pump failure. In the
current form, the SERCA assay would fail to register signif-
icanct uptake when SERCA activity is <5% of the control
value (Online Figure IV). The failure to detect differences in
SERCA uptake between working and nonworking myocar-
dium could have 2 explanations: (1) the transition to complete
working heart failure occurs with a reduction in SERCA
activity to below <5% (ie, the resolution of our measure-
ments) or (2) an event secondary to complete SERCA
inhibition is responsible for failure. Examination of the
working heart end diastolic volumes at the end point
indicated a significantly smaller end diastolic volume just
prior to failure and a significant increase in the relaxation
time (Figure 2A[iii] & 2C[iii], respectively). These data
suggest that pump failure was associated with a sustained
diastolic tone, which limited stroke volume and precipi-
tated failure of the working heart.

Measurements of cell shortening and [Ca<sup>2+</sup>]<sub>i</sub> did not show
an equivalent failure of E-C coupling during progressive TG
perfusion. Instead a new steady state was achieved after ~2
minutes of perfusion with TG and contractility was main-
tained for as long as measurements were recorded (25–30
minutes, data not shown), which is comparable to the mean
time to end point of working heart function (36 minutes) in
Protocol 1. The maintenance of Ca<sup>2+</sup> transients and cell
shortening over this time course is also consistent with the
presence of a measureable degree of contractility of the
working heart at the point of pump failure (Figure 2A[i]
dashed box). During this time, diastolic cell length decreased
indicating the development of a sustained contraction, which
may be equivalent to the sustained contraction observed in
whole hearts. This suggests that the pump fails as a result of
slow sustained increase in diastolic tone that develops after
complete SERCA inhibition and that the steady-state cellular contractile activity achieved in the complete absence of SERCA is insufficient to maintain pump function in the long term. In line with the current study, previous studies on isolated rat heart using an acute block of SERCA \[^2\text{Ca}\] and in mouse heart after SERCA knock-out \[^2\text{Ca}\] have also reported a sustained contracture and raised diastolic \([\text{Ca}^{2+}]_i\), that is associated with a raised \([\text{Na}^+]_i\) level. As suggested by both groups, the cause of raised \([\text{Na}^+]_i\) is enhanced \([\text{Na}^+]_i\) influx via NCX that occurs as a result of the increased \([\text{Ca}^{2+}]_i\) efflux required to balance enhanced \([\text{Ca}^{2+}]_i\) entry via the L-type \([\text{Ca}^{2+}]_i\) channel after SERCA block. The increased \([\text{Na}^+]_i\) would in turn reduce the effectiveness of \([\text{Ca}^{2+}]_i\) efflux via the NCX mechanism leading to a further increase in diastolic \([\text{Ca}^{2+}]_i\) and stimulation of NCX activity until a new steady state had been achieved. In addition, raised efflux of \([\text{Ca}^{2+}]_i\) from mitochondria in response to the elevated \([\text{Na}^+]_i\) may also contribute to the accumulation of \([\text{Ca}^{2+}]_i\). \[^2\text{Ca}\] The slow secondary rise of \([\text{Ca}^{2+}]_i\) is enhanced \([\text{Na}^+]_i\) influx via NCX that limits contractile activity achieved in the complete absence of SERCA and that the steady-state cellular contracture-relaxation of the heart. Circ Res. 2001;89:838–846.

Conclusions

This study shows that the relationship between SERCA activity and LV pump function in the rabbit is highly nonlinear. Intermediate levels of SERCA function (≈50% of control levels) have only minor hemodynamic effects. In the short-term, only moderate detrimental effects on LV pump function were observed despite the almost complete absence of SERCA activity. Rapid decline of function was associated with sudden sustained increase in diastolic \([\text{Ca}^{2+}]_i\). \[^2\text{Ca}\] The consequent raised diastolic \([\text{Ca}^{2+}]_i\) entry via the L-type \([\text{Ca}^{2+}]_i\) channel after SERCA block. The increased \([\text{Na}^+]_i\) would in turn reduce the effectiveness of \([\text{Ca}^{2+}]_i\) efflux via the NCX mechanism leading to a further increase in diastolic \([\text{Ca}^{2+}]_i\) and stimulation of NCX activity until a new steady state had been achieved. In addition, raised efflux of \([\text{Ca}^{2+}]_i\) from mitochondria in response to the elevated \([\text{Na}^+]_i\) may also contribute to the accumulation of \([\text{Ca}^{2+}]_i\). \[^2\text{Ca}\] The slow secondary rise of \([\text{Ca}^{2+}]_i\) is enhanced \([\text{Na}^+]_i\) influx via NCX that limits contractile activity achieved in the complete absence of SERCA and that the steady-state cellular contracture-relaxation of the heart. Circ Res. 2001;89:838–846.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- The function of the sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) in heart muscle is to ensure rapid removal of intracellular Ca\textsuperscript{2+} from the cytosol during diastole, thus allowing the heart to relax rapidly.
- After a transgenic knockout of SERCA, the adult mouse heart tolerates very low levels of SERCA; the tolerance may be due to compensatory changes in the expression and function of other Ca\textsuperscript{2+} handling proteins.
- The transgenic mouse hearts fail only when SERCA levels are <10% of normal, but the cause of contractile failure is not clear.

**What New Information Does This Article Contribute?**

- This study shows the consequences of rapid (within minutes) pharmacological inhibition of SERCA on the pump function of isolated rabbit hearts before any compensatory changes in expression of other Ca\textsuperscript{2+} handling proteins.
- Even in the absence of compensatory changes, the ventricle fails as a working pump only when SERCA activity is <5% of normal.
- The failure of pump function is the result of a sustained increase in diastolic tone as a consequence of raised diastolic Ca\textsuperscript{2+} concentration secondary to a raised intracellular [Na\textsuperscript{+}] and not due to a slower rate of relaxation.

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The extent to which SERCA activity alone determines left ventricular (LV) pump function is unknown. We show that substantial down-regulation of SERCA alone does not dramatically affect LV pump function in the rabbit myocardium, which has excitation-contraction coupling characteristics similar to humans. Secondary increases of intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} following complete SERCA inhibition eventually limit contractile function resulting in a rise in diastolic tone and LV pump failure. Intermediate levels of SERCA, comparable to those seen in failing myocardium, have minimal hemodynamic consequences. Contrary to current thinking, this work indicates that the myocardium can tolerate substantial reductions in SERCA activity before the effects of this deficiency are evident in ventricular pump function. Eventually failure occurs due to raised diastolic tone and not due to reduced rate of relaxation. This work challenges the current view of SERCA that suggests that the activity of this protein is critical for cardiac pump function because of its direct effects on excitation-contraction coupling. The implication is that improvements of human cardiac function that are a consequence of moderate increases in SERCA activity may be caused by indirect effects of long-term changes in the calcium transient.
Isolated Rabbit Working Heart Function During Progressive Inhibition of Myocardial SERCA Activity
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SUPPLEMENTAL MATERIAL

Detailed Methods

Animals
All experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). A total of 41 adult male New Zealand White rabbits weighing 3.0-3.5 kg and maintained according to national guidelines were used in the current study. Animals were euthanised with an overdose of sodium pentobarbitone (100 mg/kg) containing 0.5 ml Heparin (5000 IU/ml), administered via injection through the left marginal ear vein.

Isolated working heart studies

Working heart set-up
Schematic diagrams of the working heart setup are shown in Online Figure I in both working heart (Figure I(i)) and non-working heart (retrograde perfusion, Figure I(ii)) modes. Hearts were excised via a thoracotomy and submerged in a modified Tyrode’s solution at 4 °C. The modified Tyrode’s solution contained the following (in mmol/L): NaCl (116), NaHCO3 (20), Na2HPO4 (0.4), MgSO4 (1), KCl (4), CaCl2 (2.5) and Glucose (11).

The experimental apparatus used for the isolated working heart experiments was based upon a system previously described by Neely and colleagues (1967)1. Hearts were cannulated via the aorta and perfused with modified Tyrode’s solution at 37 °C gassed with 95 % O2 – 5 % CO2 mixture to maintain pH at 7.4 initially in non-working heart mode under a constant pressure of 60 mmHg. The left atrium was cannulated via a pulmonary vein, with the remaining pulmonary veins tied off to prevent any leaks. Perfusion was then switched to working heart mode, and a fixed volume of 540 ml was re-circulated through a 5 µm pore in-line filter for the remainder of the experiment. Preload was set at 6 mmHg and afterload, dictated by a column of fluid above the aortic valve, was set at 60 mmHg. A compliance chamber partially filled with 15 ml of air was located in the aortic outflow line to provide an aortic Windkessel. An in-line ultrasonic flowmeter (Malema, FL) was located between the aortic cannula and compliance chamber for continuous monitoring of aortic flow (AoF). A pair of platinum electrodes were positioned on the right atrium for pacing. These were connected to a constant voltage isolated stimulator. Hearts were paced at mean rates of 206±9 and 216±1 beats/min throughout Protocols 1 and 2 respectively. A pressure-volume (PV) catheter was fed through a plastic haemostasis valve located above the aortic cannula and advanced past the aortic valve into the left ventricle for continuous measurement of haemodynamic function. In the isolated working heart system used in this study, stroke volume is defined as the sum of coronary flow and AoF. Therefore AoF is the proportion of the cardiac output which does not return to the coronary circulation.

Left ventricular pressure-conductance measurement
A custom-built 3F variable segment PV catheter was used for continuous measurement of cardiac contractile function (Scisense Inc., Canada). A high fidelity pressure sensor located close to the tip of the catheter was surrounded on either side by several ring electrodes; two at the tip, and five preceding the pressure sensor. The outermost electrodes acted as excitation and ground reference respectively, while the sensing portion of the conductance catheter was determined by the distance between the inner electrode at the tip, and a second inner sensing electrode located proximal to the tip of the catheter. The PV control unit (VSL-0012) allowed selection of 11.0, 14.0, 17.0 or 19.0 mm sensing portions. Through application of a constant current via the excitation electrode an electric field was generated within the left ventricle and recorded by the inner sensing electrodes. Prior to the start of experiments, the catheter was placed sequentially in two of a series of machine-drilled cylindrical cuvette wells (radius of 4.0 mm and
filled with Tyrode’s solution at 37 °C. A linear relationship between the raw analogue signal generated by the catheter and the absolute volume was established using the following equation for calculating the volume of a cylinder:

\[ V = \pi r^2 L \]  

(1)

Where \( V \) is the cylinder volume, \( r \) is the radius of the cylinder (mm) and \( L \) is the length of the sensing portion of the catheter (mm). As this calibration method accounts for both solution resistivity and sensing electrode distance, relative volume was converted to absolute volume using the following equation:

\[ V_T = \frac{1}{\alpha} (V_C - V_P) \]  

(2)

Where \( V_T \) is the true volume (ml), \( \alpha \) is a dimensionless constant used to correct the volume signal for non-uniformity of the electric field, \( V_C \) is the volume measured by the catheter (ml) and \( V_P \) is the volume owed to structures other than the perfusate within the left ventricle (ml; parallel conductance).

**Calculation of parallel conductance (\( V_P \)) and alpha (\( \alpha \))**

\( V_P \) was estimated by injection of a small quantity of 20 % saline solution into the preload line resulting in a transient period (approximately 6-7 beats) where \( V_C \) was changing without any alteration in left ventricular pressure. From the resultant beats the maximum and minimum volume values were plotted to establish a linear relationship. This relationship was then extrapolated to the point where it crosses the line of identity, where maximum and minimum volumes are equal. Where these two lines meet left ventricular volume is 0, and the volume measured by the catheter at this point is \( V_P \). The dimensionless constant \( \alpha \) represents the ratio of the conductance catheter-derived stroke volume to the independently measured true stroke volume. Calculation of \( \alpha \) in the isolated working heart experiments was achieved by independent measurement of cardiac output, obtained by summation of AoF and coronary flow. AoF was measured throughout the experiment with the use of an in-line ultrasonic flow meter, whilst coronary flow was measured at regular intervals by timed collection of coronary effluent from the heart.

**Experimental Protocol**

**i) Protocol 1 (>5 min):** Following a 5 min steady state period in working heart mode, hearts were perfused with either 3 µmol/L thapsigargin (TG; \( n = 6 \)) or vehicle (DMSO; \( n = 5 \)). For hearts treated with TG, left ventricular function was continuously recorded until AoF fell to undetectable levels (<20 mls/min, lower limit of resolution of the in-line flow meter). Visual inspection of the overflow from the afterload chamber in the working heart system confirmed significant reduction in forward flow. Online Figure II shows the timecourse of complete cessation of AoF following endpoint. At this point perfusion was switched to non-working heart mode (retrograde perfusion) and hearts were perfused for 2 min with a nominally Ca\(^{2+}\)-free Tyrode’s solution containing 50 µmol/L ethylene glycol tetraacetic acid (EGTA) followed by perfusion for 0.5 min with 3 µmol/L thapsigargin. For vehicle treated hearts, LV function was recorded continuously for time-matched durations, after which non-working heart mode was established and hearts were perfused with nominally Ca\(^{2+}\)-free Tyrode’s solution containing 50 µmol/L EGTA then 3 µmol/L thapsigargin as above. Mean endpoint data were taken from the 4 cycles immediately following the drop of AoF to undetectable levels (< 20 mls/min).

**ii) Protocol 2 (<5 min):** For correlation of working heart contractile function with SR Ca\(^{2+}\) uptake, isolated working hearts were exposed to 3 µmol/L TG (\( n = 10 \)) for short periods of time (1-4 min). Percentage change in steady state contractile function was monitored following perfusion with TG until dP/dt\(_{\text{max}} \) had fallen to between 5-22 % of control values. At this point perfusion was switched to non-working heart mode and hearts were perfused with nominally Ca\(^{2+}\)-free Tyrode’s containing 50 µmol/L EGTA for 2 min. Vehicle hearts perfused with DMSO (\( n = 7 \)) were time-matched before being subject to the 2 min non-working heart mode perfusion with EGTA.
**Tissue homogenisation**

Following 2 min perfusion with nominally Ca\(^{2+}\) free Tyrode’s solution containing 50 µmol/L EGTA (and TG in the Protocol 1 group), the PV catheter was removed from the left ventricular cavity and a section of left ventricular free wall weighing approximately 1.5 g was rapidly cut from the heart and any excess fatty tissue removed. The final sample was weighed before being snap frozen in liquid nitrogen. Tissue was roughly chopped with a scalpel blade before being pulverised with a mortar and pestle. Five-times volume of ice-cold homogenisation buffer was gradually added during this time, and tissue was homogenised using an Ultraturrax T8 homogeniser until no solid tissue pieces remained. Homogenisation buffer containing a protease/phosphatase inhibitor cocktail was prepared in 10 mmol/L imidazole (pH 7) and contained the following (in mmol/L): NaF (10), Na\(_3\)VO\(_4\) (1), NaPP (2), β-GP (2) and Calyculin A (0.05). The final homogenate was aliquoted into 1.5 ml Eppendorf tubes and stored at -80 °C prior to subsequent biochemical analysis.

**SR Ca\(^{2+}\) uptake studies**

Total protein content of the LV homogenate was determined using a Bradford protein assay. Following this, homogenate containing 6 mg of total protein was re-suspended in 1.5 ml of mock intracellular solution, placed in a cuvette and stirred to maintain it in suspension (4 mg/ml total protein, 20-22 °C). The mock intracellular solution contained the following (in mmol/L): KCl (100), Na\(_2\)-ATP (5), Na\(_2\)-phosphocreatine (10), MgCl\(_2\) (5.5), HEPES (25), K\(_2\)-EGTA (0.05), pH 7.0. 20 mmol/L Oxalate was added to the cuvette to maintain low and constant levels of intra-SR [Ca\(^{2+}\)]. Ruthenium Red (RuR; 7 µmol/L) was added to block SR Ca\(^{2+}\) efflux via the ryanodine receptor and inhibit mitochondrial Ca\(^{2+}\) uptake. 10 µmol/L Fura-2 free acid was used to monitor [Ca\(^{2+}\)] within the cuvette using a dual-wavelength spectrophotometer (Cairn Research, U.K.). The time course of the decrease in [Ca\(^{2+}\)] within the cuvette was monitored following the addition of an aliquot of CaCl\(_2\). In each cuvette following a series of uptake measurements, the Fura-2 signal was calibrated in situ using two standard solutions which buffered [Ca\(^{2+}\)] using 10 mmol/L total [EGTA] to the following levels: (i) <1 nmol/L [Ca\(^{2+}\)] to provide a minimum ratio value (R\(_{min}\)) and (ii) 375 nmol/L [Ca\(^{2+}\)] to provide a second calibration signal (R\(_{375}\)). The value of (β x K\(_d\)) used in this study to calculate the [Ca\(^{2+}\)] within the cuvette was 1.66 µmol/L determined in our cuvette system using calibration solutions (i.e. mock intracellular solutions +10 mmol/L EGTA). The β value for our Fura-2 measurement system was 9.2, suggesting a K\(_d\) for Fura-2 of 181 nM (which is very close to the value reported by Grynkiewicz et al., 1985). The Fura-2 calibration was verified in the presence of 4mg/ml homogenate and we could detect no significant difference in the (β x K\(_d\)) value (=1.59±0.08, n=4 hearts) confirming the accuracy of the [Ca\(^{2+}\)] conversions in this study. Increasing total protein concentration of the homogenates from the TG group 5 fold also failed to result in a measurable uptake component (data not shown). The sensitivity of the assay was verified and is shown in Online Figure IV. The effects of residual, unbound TG and regional differences in TG perfusion in the TG group homogenates are shown in Online Figures V and VI respectively.

**Left ventricular myocyte isolation**

Hearts were excised via a thoracotomy and submerged in a modified Krebs-Henseleit (KH) solution of the following composition (mmol/L): NaCl (130), KCl (4.5), HEPES (5), NaH\(_2\)PO\(_4\) (0.4), MgCl\(_2\) (3.5) and Glucose (10), pH 7.25 at 37 °C with NaOH. Hearts were removed and perfused retrogradely at 25 ml.min\(^{-1}\) (37 °C) with a modified KH solution containing 0.75 mmol/L [Ca\(^{2+}\)] for 5 min, followed by a nominally Ca\(^{2+}\)-free KH solution with 0.1 mmol/L EGTA for 5 min. Hearts were then perfused with KH solution containing 0.24 mmol/L [Ca\(^{2+}\)], 1 mg/ml collagenase (type I) and 0.06 mg/ml protease (type XIV). After ~4-5 min, enzyme was removed and the left ventricular free wall was then cut into strips in the re-circulated enzyme solution containing 1 % bovine serum albumin (BSA) before being mixed to yield a single cell suspension. Cells were maintained in either Ca\(^{2+}\)-free KH solution or 1 mmol/L Ca\(^{2+}\) (via stepwise increments) until use.
Myocyte field stimulation with simultaneous whole-cell epifluorescence and cell-shortening measurements

Intact cardiomyocytes in 1 mmol/L modified Ca\textsuperscript{2+} KH solution were loaded with a Ca\textsuperscript{2+} sensitive fluorophore (5 μmol/L Fura-2AM) by incubation for ~10 min. The incubation medium was removed and the cells re-suspended in a second modified KH solution containing 2.5 mmol/L [Ca\textsuperscript{2+}]. Cells were incubated for a further 30 min to ensure complete de-esterification. Cardiomyocytes were allowed to settle on a coverslip in a bath (Cell Microcontrols, VA) and super-fused with the same modified KH solution at 37 °C. Cells were field stimulated at a frequency of ~3.5 Hz with 2 ms duration voltage pulses delivered through parallel platinum wires (stimulation voltage set to 1.5 times the threshold). Cells were perfused with TG for between 5 and 240s (final TG concentration of 3 μmol/L) followed by rapid application of 15 mmol/L caffeine to cause complete release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR). The amplitudes of the caffeine-induced Ca\textsuperscript{2+}-transients were used as a measure for SR Ca\textsuperscript{2+} content. A parallel set of vehicle (DMSO) time control experiments were performed to achieve control SR Ca\textsuperscript{2+} contents. Fura-2AM fluorescence (340 & 380 nm excitation; R\textsubscript{340/380nm}) was measured using a spinning wheel spectrophotometer (Cairn Research Ltd., U.K.; sampling rate of 500 Hz) whilst cellular shortening was measured using a video edge detection system (IonOptix, MA; sampling rate of 200 Hz). Data were analysed offline (OriginPro v6.1); Fura-2AM fluorescence ratio was converted to intracellular [Ca\textsuperscript{2+}] (\([\text{Ca}^{2+}]_i\)) as previously described\textsuperscript{2,3}. 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.

Assessment of intracellular Na\textsuperscript{+} (\([\text{Na}^+]_i\)) and the accompanying cell shortening) was performed as above in a separate population of cells loaded with the Na\textsuperscript{+} sensitive fluorophore sodium-binding benzofuran isophthalate (SBFI-AM, 10 μmol/L with 0.02 % pluronic acid for 1 hr at RT). Following de-esterification, cells were perfused with modified KH solution containing 2.5 mmol/L [Ca\textsuperscript{2+}] and field stimulated at ~3.5 Hz. Perfusion was switched to 3 μmol/L TG for 240 s followed by cessation of electrical stimulation and continued recording for 100 s. SBFI fluorescence was acquired with excitation at 340 & 380 nm and emission at > 500nm. Data were analysed offline (OriginPro v6.1). The SBFI fluorescence ratio was converted to [Na\textsuperscript{+}] using previously published calibration values (from Donoso et al. (1992)\textsuperscript{4} and Maier et al. (1997)\textsuperscript{5}) and assuming a [Na\textsuperscript{+}] prior to application of TG of 8.6 mmol/L (from Despa et al. (2002)\textsuperscript{6}, measured at 3Hz in the rabbit). Shortening data were acquired simultaneously and analysed as above.

Online Figure legends

Online Figure I. Working heart experimental set-up

Schematic diagrams of the rabbit working heart experimental set-up depicting perfusion in (i) non-working heart mode (retrograde perfusion) and (ii) working heart mode. In each mode, systemic flow is shown by the red lines, the overflow is shown by blue lines; solution reservoirs were gassed to maintain pH, B1 and B2 represent bubble traps in the system.

Online Figure II. Timescale of cessation of aortic flow

In protocol 1, endpoint was defined at the point at which AoF was undetectable (<20 mls/min on the inline flow meter). Volume data from the PV catheter were used to calculate cardiac output (stroke volume * heart rate) over a number of beats following endpoint in Protocol 1. Coronary flow at endpoint was calculated by subtracting the aortic flow measured by the flow meter at its final point of resolution (20 mls/min) from the cardiac output immediately prior to endpoint. The accuracy of the catheter in estimating coronary flow was confirmed by comparison with timed measurement of coronary effluent from the heart. The point at which cardiac output dropped below the mean coronary flow at endpoint (i.e. when AoF reaches 0 mls/min) is shown by the dotted line. Once cardiac output is less than coronary flow, the heart can no longer support the pressure head of fluid which dictates afterload, and function will
decline due to the drop in afterload. This demonstrates that complete cessation of AoF occurred within 10 beats (approx. 3 seconds) of the endpoint thereby confirming the resolution of the measurements made with respect to the absolute endpoint of whole heart forward flow (0 mls/min AoF).

**Online Figure III.** Comparison of Ca$^{2+}$ uptake in vehicle treated hearts.

Ca$^{2+}$ uptake was compared between vehicle treated hearts subject to Protocols 1 and 2 (4mg/ml) to determine the potential effect of the additional 30 s TG wash following endpoint in Protocol 1. SERCA activity was quantified at 0.3, 0.5 and 1 µmol/L from the declining phase of free [Ca$^{2+}$]. No alteration in the gradients of Ca$^{2+}$ uptake was detected between the two vehicle treated groups confirming a lack of effect of the additional TG wash step in Protocol 1 vehicle hearts. In addition, the maximum uptake rate ($V_{max}$) was not significantly different between the groups (Protocol 1: 330.2±0.6 nmoles/mg/min vs. Protocol 2: 282.2±0.2 nmoles/mg/min).

**Online Figure IV.** Sensitivity of the Ca$^{2+}$ uptake assay.

Superimposed traces of Fura-2 fluorescence ratios following addition of CaCl$_2$ to 6 cuvettes containing homogenate samples of varying total protein amounts (0.125 – 6 mg as indicated, normalised to peak fluorescence following CaCl$_2$ addition; final volume was 1.5 mls in all cuvettes) from a vehicle treated (DMSO) heart and a TG perfused heart both subject to Protocol 1. Reducing total protein amount of vehicle treated sample to between 3 and 0.125 mg, consistently showed detectable Ca$^{2+}$ uptake whilst in contrast all TG treated homogenates from Protocol 1 demonstrated undetectable uptake (grey trace). These results suggest that SERCA activity in TG treated hearts was reduced more than 40 fold when compared to vehicle treated hearts.

**Online Figure V.** Effect of residual unbound TG on Ca$^{2+}$ uptake.

In order to determine if there was contamination of TG treated samples with residual unbound TG, mixed samples with equal amounts (3 mg) of TG and vehicle treated (DMSO) hearts were assayed in the same cuvette (black trace). Traces for 6 mg TG treated and 6 and 3 mg vehicle treated are superimposed for reference. Detectable uptake was maintained in mixed samples. This was slowed when compared with that of 3 mg DMSO treated alone (red trace) most probably reflecting the higher buffering of Ca$^{2+}$ due to the higher amount of total protein present in the mixed sample. Final volume was 1.5 mls in all cuvettes.

**Online Figure VI.** Regional inhibition of SERCA during TG perfusion.

An assessment of the regional inhibition of SERCA during TG perfusion of the whole heart was performed by taking additional samples from the apical free LV wall in 2 hearts perfused with TG for 100 s in Protocol 2. SERCA activity was quantified at 0.3, 0.5 and 1 µmol/L from the declining phase of free [Ca$^{2+}$]. No alteration in the gradients of Ca$^{2+}$ uptake was detected between regional samples indicating homogeneous perfusion of the LV with TG.

**Online Figure VII.** Changes in [Na$^+$], during TG perfusion.

Typical trace of SBFI fluorescence in an isolated rabbit ventricular myocyte field stimulated at ~3.5 Hz. Application of TG (3 µmol/L) and the point at which electrical stimulation was stopped are indicated by the bars above. The mean SBFI fluorescence ratio was significantly increased from 0.631±0.011 (pre-addition) to 0.637±0.010 following 240 s in TG ($P<0.05$, $n=10$ cells from 5 hearts). Assuming a control [Na$^+$], of 8.6 mmol/L this predicts an increase to 12.0±1.0 mmol/L during TG which was accompanied by a significant reduction in cell shortening amplitude (to 51% of pre-addition). At 100 s post cessation of stimulation, the SBFI ratio dropped to 0.623±0.010 ($P<0.05$ vs. TG+240s; predicting a decrease of [Na$^+$], to 5.7±2.8 mmol/L) as a consequence of sarcolemmal extrusion.
Reference List


Online Figures

(i) Non-working heart mode

- Systemic Flow
- Overflow

(ii) Working heart mode

- Systemic Flow
- Overflow

CO (ml/min)

<table>
<thead>
<tr>
<th>No. of beats post endpoint</th>
</tr>
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<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>10</td>
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</tbody>
</table>

Free [Ca^{2+}] (μM)

<table>
<thead>
<tr>
<th>Gradient of decline in free [Ca^{2+}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1 Vehicle</td>
</tr>
<tr>
<td>0.5</td>
</tr>
</tbody>
</table>

Mean CF (endpoint)

Gradient of decline

Protocols 1 and 2 Vehicle
IV

Normalised Fluorescence

<table>
<thead>
<tr>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
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</thead>
</table>

25 s

V

Normalised Fluorescence

<table>
<thead>
<tr>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
</tr>
</thead>
</table>

25 s

VI

% of vehicle uptake

| 0 | 20 | 40 | 60 | 80 |

Free \([Ca^{2+}]\) (\(\mu M\))

- heart 1 apical sample
- heart 1 mid/basal sample
- heart 2 apical sample

70 s

VII

SBFI fluorescence (340/380 nm)

| 0.60 | 0.61 | 0.62 | 0.63 |

3 \(\mu M\) TG

Stim Off

50 s