ION CHANNELS, RECEPTORS AND TRANSPORTERS

Enhanced Ca^{2+} influx through cardiac L-type Ca^{2+} channels maintains the systolic Ca^{2+} transient in early cardiac atrophy induced by mechanical unloading

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Abstract Cardiac atrophy as a consequence of mechanical unloading develops following exposure to microgravity or prolonged bed rest. It also plays a central role in the reverse remodelling induced by left ventricular unloading in patients with heart failure. Surprisingly, the intracellular Ca^{2+} transients which are pivotal to electromechanical coupling and to cardiac plasticity were repeatedly found to remain unaffected in early cardiac atrophy. To elucidate the mechanisms underlying the preservation of the Ca^{2+} transients, we investigated Ca^{2+} cycling in cardiomyocytes from mechanically unloaded (heterotopic abdominal heart transplantation) and control (orthotopic) hearts in syngeneic Lewis rats. Following 2 weeks of unloading, sarcoplasmic reticulum (SR) Ca^{2+} content was reduced by ~55 %. Atrophic cardiac myocytes also showed a much lower frequency of spontaneous diastolic Ca^{2+} sparks and a diminished systolic Ca^{2+} release, even though the expression of ryanodine receptors was increased by ~30 %. In contrast, current clamp recordings revealed prolonged action potentials in endocardial as well as epicardial myocytes which were associated with a two to fourfold higher sarcolemmal Ca^{2+} influx under action potential clamp. In addition, Cav1.2 subunits which form the pore of L-type

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Ca^{2+} channels (LTCC) were upregulated in atrophic myocardium. These data suggest that in early cardiac atrophy induced by mechanical unloading, an augmented sarcolemmal Ca^{2+} influx through LTCC fully compensates for a reduced systolic SR Ca^{2+} release to preserve the Ca^{2+} transient. This interplay involves an electrophysiological remodelling as well as changes in the expression of cardiac ion channels.

Introduction

Cardiac atrophy can be observed during prolonged bed rest, space flight or cachexia [19]. It also plays a central role in the reverse remodelling induced by left ventricular unloading in patients with heart failure [19]. In contrast to hypertrophic remodelling, cardiac atrophy is generally not well understood. Intracellular Ca^{2+} handling plays an essential role in regulating cardiac function and is pivotal to adaptations in hypertrophic cardiac remodelling [18, 19]. An early cellular response to a hypertrophic stimulus is the prolongation of ventricular action potentials (AP) which greatly facilitates Ca^{2+} influx via L-type Ca^{2+} channels (LTCC) [10, 48, 53]. In addition, phosphorylation of phospholamban (PLB) is increased, thus relieving the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) from its inhibition [7, 28]. As a consequence, Ca^{2+} content of the SR and systolic Ca^{2+} release from the SR are enhanced [3, 5, 16, 31, 53]. Collectively, these alterations cause larger systolic Ca^{2+} transients which are thought to allow adaptation to the increased cardiac workload in early hypertrophy [9, 11, 17, 43, 47]. We have previously reported a reduced phosphorylation of several phosphoproteins, including PLB, in cardiac atrophy [41]. Unphosphorylated PLB exerts a stronger inhibitory effect on SERCA which should lead to a reduced SR Ca^{2+} load. Hence, one may expect that Ca^{2+} release during systole is diminished and amplitudes of systolic Ca^{2+} transients are dampened in atrophic hearts. The shape of the systolic Ca^{2+} transient, however, was found to be unaltered in early atrophic remodelling [22, 46].

The mechanisms underlying the maintenance of the Ca^{2+} transient in early cardiac atrophy are unresolved. Short-term cardiac unloading causes a marked prolongation of ventricular APs and a higher density of the L-type Ca^{2+} current [40]. Accordingly, a reduced systolic Ca^{2+} release from the SR may be compensated by a concurrent upregulation of the Ca^{2+} inflow from the extracellular space in atrophic cardiac myocytes. To test this hypothesis, we determined the effect of cardiac atrophy induced by mechanical unloading on key components of myocardial Ca^{2+} cycling.

Methods

Animal model

Cardiac atrophy was induced in syngeneic male Lewis rats (270±5 g, n=34, Charles River, Sulzfeld, Germany) by heterotopic abdominal heart transplantation as previously described [12, 14, 21, 22, 29, 40, 41, 45, 46]. The donor heart was harvested under deep anaesthesia (thiopental-sodium, 100 mg/kg body weight) and was transplanted into the abdominal cavity of the recipient rat under isoflurane anaesthesia (2–2.5 %). Perioperative pain management was performed using buprenorphine (0.1 mg/kg body weight) and metamizole-sodium (300 mg/kg body weight). The aortic and pulmonary vessels of the donor heart were anastomized infrarenally to the abdominal aorta and the inferior vena cava of the recipient animal, respectively. In this configuration, coronary perfusion is conserved and left ventricular filling is substantially reduced. Spontaneous contraction returned within minutes after reperfusion and was checked regularly. The transplanted (atrophic) and the orthotopic (control) hearts of the recipient animal were removed in deep anaesthesia (thiopental-sodium, 100 mg/kg body weight) 2 weeks after transplantation. An unloading period of 2 weeks was chosen to induce a stable cardiac atrophy in accordance with previous studies [12, 14, 40, 41] while avoiding the detrimental effects of prolonged unloading on cardiac excitation–contraction coupling and contractility [21, 22, 29, 45]. For all experiments, the orthotopic hearts served as corresponding controls. Animal experiments were conducted in accordance with institutional guidelines and were approved by local authorities (Ministry of Science and Health, Hamburg, Germany, permit number 02/04). Furthermore, they complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe No. 123).

Isolation of cardiomyocytes

For single-cell experiments, cardiac myocytes were enzymatically isolated from the control or the atrophic heart of one animal. Since the time span between sacrificing the animal and the beginning of the perfusion using the Langendorff apparatus must be kept at a minimum to ensure isolation of cardiac myocytes of high quality, we randomly assigned either the orthotopic or the transplanted heart to the cell isolation procedure. The other heart was discarded. Cardiac myocytes were isolated from the endocardial and epicardial layer of the center part of the left ventricular free wall [15, 40, 51, 52]. For experiments depicted in Fig. 3b, myocytes were isolated from control male Lewis rats (270 g,

$n=3$). Following isolation, cells were stored in a Ca^{2+}-free modified Tyrode's solution. Only single rod-shaped cells with clear cross-striation and no spontaneous contractions were used for the experiments within 6 h following the isolation. All experiments were performed at a temperature of 21–24 °C.

Ca²⁺ epifluorescence measurements

Ca^{2+} epifluorescence was recorded as previously described [34]. Cells were incubated in an extracellular solution (containing [mmol/l]: NaCl 135, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 1.2, Hepes 10, glucose 20, $CaCl_2$ 1.25) with Fura-2-AM (4 μmol/l). Cells were transferred into a chamber (Cell MicroControls, Norfolk, VA, USA) mounted on an inverted microscope (Nikon Eclipse TS100). Ca^{2+} transients of intact myocytes were recorded during field stimulation (0.5 Hz, 4 ms duration; MyoPacer, IonOptix Corporation, Milton, MA, USA). Cells were alternatively excited at 340 and 380 nm (hyper-switch dual excitation, IonOptix Corporation). The F_{340}/F_{380} ratio was used as an index of cytosolic Ca^{2+} concentration. In some experiments, thapsigargin was washed in and after an incubation time of 5 min, Ca^{2+} transients were assessed using Fluo-3-AM (10 μmol/l, F/F_0 from excitation at 480±15 nm and emission at 535±20 nm). Ca^{2+} transients were analysed using Ionwizard (v. 5.0, IonOptix Corporation).

Cellular electrophysiology

The ruptured-patch whole-cell configuration was used with an EPC-9 amplifier controlled by the Pulse software (Heka Elektronik, Lambrecht, Germany) [40, 51]. Membrane capacitance and series resistance were calculated using automated procedures of the EPC-9. Whole-cell currents and membrane potentials were low pass-filtered at 1 kHz, sampled at 5 kHz and analysed using the Pulse-fit software (v. 8.80, Heka Elektronik) and custom-made procedures in IgorPro (v. 6.2, Wavemetrics, Lake Oswego, OR, USA). Patch pipettes were pulled from borosilicate glass (GC150-15, Harvard Apparatus, Holliston, MA, USA). Pipette resistance averaged 2.2±0.1 MΩ ($n=49$) in the SR release experiments, 2.7±0.1 MΩ ($n=75$) in the I_{NCX} experiments and 3.4±0.1 MΩ ($n=50$) in the AP clamp experiments. The series resistance was between 3.0 and 8.9 MΩ and was compensated by 85 %. Sarcoplasmic reticulum (SR) release experiments were performed using a CsCl-based pipette solution (mmol/l): CsCl 128, $MgCl_2$ 5 TEA-Cl 20, EGTA 0.1, Hepes 10, Mg_2-ATP 2, titrated to pH=7.20 with CsOH. Extracellular solution (modified Tyrode's solution) contained (mmol/l): NaCl 138, KCl 4, $MgCl_2$ 1, NaH_2PO_4 0.33, $CaCl_2$ 2, glucose 10, Hepes 10, titrated to pH=7.30 with NaOH. For inhibition of K^+ currents, 4 mmol/l 4-aminopyridine and 0.1 mmol/l $BaCl_2$ were added to the bath solution [49, 54]. At

the beginning of the experiment, cells were repeatedly stepped from a holding potential of −90 to 0 mV (500 ms) at a frequency of 0.5 Hz over 60 s to obtain steady-state filling of the SR. For measurement of I_{NCX}, the pipette solution contained (mmol/l): CsCl 85, glucose 5.5, EGTA 14, $MgCl_2$ 3, $CaCl_2$ 3.92, Hepes 10, NaCl 15, ATP 2, titrated to pH=7.20 using CsOH (total Cs, 130 mmol/l). Cells were patched in a K^+-free Tyrode solution. To activate I_{NCX}, extracellular solution was switched to a Li^+-based solution containing (mmol/l): LiCl 140, $MgCl_2$ 1, NaH_2PO_4 0.33, $CaCl_2$ 2, glucose 10, Hepes 10, titrated to pH=7.30 using LiOH. I_{NCX} was quantified as the difference between the holding current and the maximal outward current when the current amplitude reached a steady state. For AP clamp experiments, a K-glutamate pipette solution containing (mmol/l) K-glutamate 120, KCl 10, $MgCl_2$ 2, EGTA 10, Hepes 10, Na_2-ATP 2, and titrated to pH=7.20 with KOH was used in combination with a modified Tyrode's solution. Ca^{2+} influx was estimated using 30 μmol/l Cd^{2+}. At this rather low concentration, Cd^{2+} inhibits $I_{Ca,L}$ but only marginally affects other currents such as I_{to} and I_{NCX} [1, 20]. Moreover, more specific inhibitors of $I_{Ca,L}$, such as dihydropyridines or D600, are potent inhibitors of I_{to} and would thus interfere with the estimation of Q_{Ca}. Overall, the AP-induced Cd^{2+}-sensitive current yields a reasonable estimate for the AP-induced Ca^{2+} influx [35, 51, 53]. All currents and Ca^{2+} charges were normalised to cell capacitance.

Ca²⁺ sparks

Ca^{2+} sparks were assessed by standard confocal microscopy using a Zeiss Axiovert 200 M microscope and the Zeiss LSM5 image system (Carl Zeiss AG, Oberkochen, Germany) [55]. The bath solution consisted of a modified Tyrode's solution containing 2 mmol/l $CaCl_2$. Cells were loaded with 10 μg/ml Fluo-4-AM (Invitrogen, Darmstadt, Germany). Following an initial phase of field-stimulated pacing (60 s, 0.5 Hz, 4 ms duration), spontaneous Ca^{2+} release was recorded over 30 s using a line scan, with the scanning line placed parallel to the longitudinal axis of the cell at a central focal plane. Spark frequency was quantified using the sparkmaster plugin in ImageJ (Rasband W, NIH, Bethesda, MD, USA) [33].

Western blot analysis

For determination of protein expression, the transplanted as well as the orthotopic hearts were excised in deep anaesthesia (see earlier discussion). Left ventricular tissue was immediately shock-frozen and stored at −80 °C for later analysis. Western blotting was performed as described previously [41] with primary antibodies against ryanodine receptor (RyR, 1:10,000, Sigma-Aldrich), S^{2843}-phosphorylated RyR (1:5,000, Badrilla),

Cav1.2 (1:200, Santa Cruz), calsequestrin (CSQ, 1:2,500, Dianova) and GAPDH (1:20,000, Biotrend).

Statistical analysis

All data are given as mean±SEM. When only two groups were compared, statistical significance was calculated by Student's t-test or a Mann–Whitney test as appropriate. Multiple comparisons were performed using a one-way ANOVA followed by Newman–Keuls post hoc test or a repeated-measures two-way ANOVA using PRISM (v. 5.0, GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as $P<0.05$.

Results

Systolic Ca^{2+} transients

Systolic Ca^{2+} transients were investigated in Fura-2-AM-loaded isolated cardiac myocytes under field stimulation. In myocytes isolated from control hearts, no layer-specific differences between endocardial and epicardial cardiac myocytes regarding shape and duration of intracellular Ca^{2+} transients were observed (data not shown). Therefore, Ca^{2+} transients were analysed in cell suspensions pooled from endocardial and epicardial myocytes to increase statistical power. Ca^{2+} transients did not differ between atrophic and control myocytes (Fig. 1a). In particular, the amplitude of the Ca^{2+} transients (Fig. 1b), the diastolic Ca^{2+} concentration (Fig. 1c), as well as the time course of the Ca^{2+} transients (Fig. 1d, e) were not significantly different. These findings confirm that the intracellular Ca^{2+} transients remain unchanged in early cardiac atrophy [22, 46].
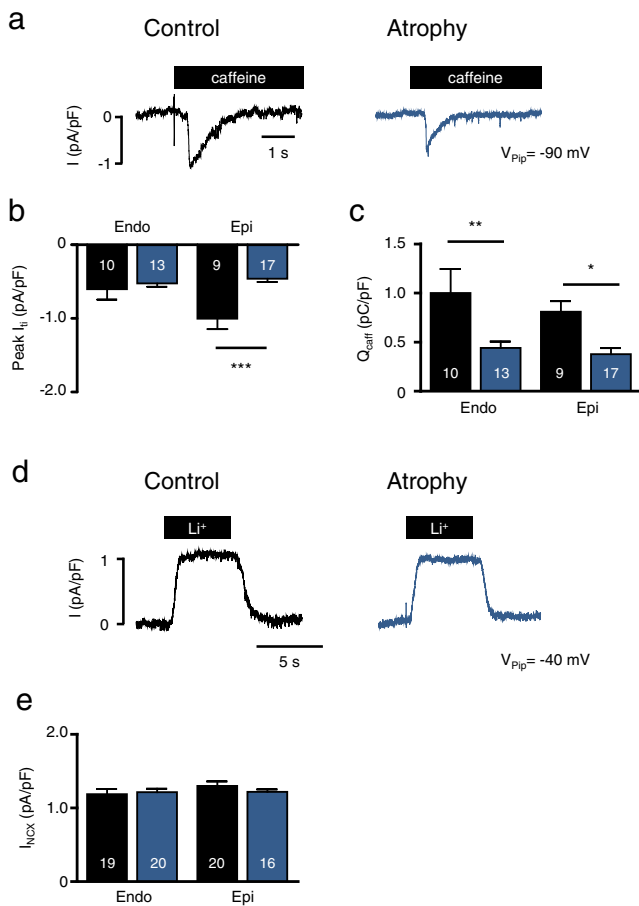
SR Ca^{2+} content

We have previously reported a reduced phosphorylation of PLB in atrophic cardiac myocytes [41] which predicts a lower SERCA activity and consequently a reduced SR Ca^{2+} load in atrophic hearts. To test this prediction, we quantified the amount of the steady-state SR Ca^{2+} content in isolated cardiac myocytes using the patch clamp technique. To assess the degree of cellular atrophy and to allow for normalisation to the reduced cell size, cell capacitance was determined at the beginning of each experiment. Consistent with previous reports, cell capacitance was significantly lower in atrophic than in control cardiac myocytes (123±3 pF, n=96 vs. 161±5 pF, n=98, $p<0.0001$) [40]. At a holding potential of $V_{Pip}=-90$ mV, Ca^{2+} release from the SR was induced by rapid superperfusion of the investigated cardiac myocyte with 10 mmol/l caffeine [30, 38, 49, 50,
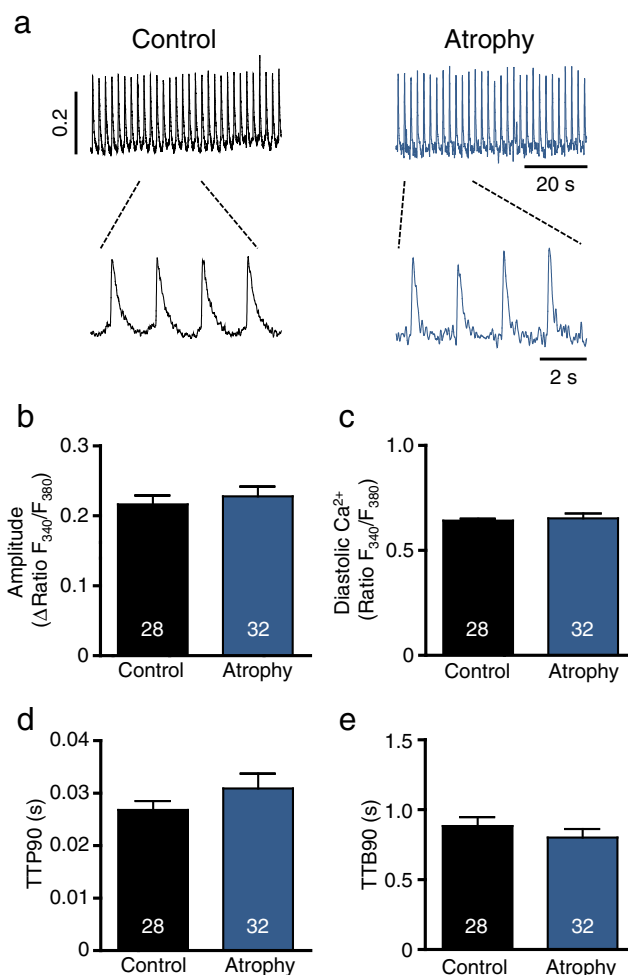
Fig. 1 Preserved systolic Ca^{2+} transients in myocytes of atrophic hearts. a Representative Ca^{2+} transients from left ventricular myocytes isolated from control (black spectrum, left panel, n=5) and atrophic (blue spectrum, right panel, n=5) hearts. b–e Summary data for the main parameters of the Ca^{2+} transients. Numbers in columns represent the number of individual myocytes. TTP90 time to 90 % peak, TTB90 time to 90 % baseline

54]. In the presence of caffeine, Ca^{2+} is released from the SR, cannot be stored in the SR again and is therefore completely removed from the cytoplasm by the Na^+–Ca^{2+} exchanger [8, 27]. This results in the activation of a transient inward current (I_{ti}) following caffeine application (Fig. 2a). In myocytes isolated from atrophic hearts, the amplitude of this current (peak I_{ti}) was significantly lower than in control myocytes (~−10 % in endocardial and~−50 % in epicardial myocytes; Fig. 2b). More importantly, the integral of I_{ti} (Q_{caff}), which reflects the total amount of Ca^{2+} released from the SR [49], was significantly smaller (~−55 %) in the atrophic cardiac myocytes (Fig. 2c). These observations indicate that the amount of Ca^{2+} stored in the SR is markedly reduced in early atrophy.

Fig. 2 Decreased SR Ca^{2+} content and unaltered I_{NCX} in myocytes of atrophic hearts. **a** Representative whole-cell current traces recorded from myocytes of control (*black spectrum, left panel, n=6*) and atrophic (*blue spectrum, right panel, n=6*) hearts. At a pipette potential of −90 mV, caffeine (10 mmol/l) was washed in to induce SR Ca^{2+} release, resulting in a transient inward current (I_{ti}). **b** Average values of the peak current density of I_{ti}. **c** total amount of Ca^{2+} transported by I_{ti} upon caffeine application (Q_{caff}). Q_{caff} was calculated as the area under the curve of I_{ti}. **d** Representative whole-cell current traces recorded from left ventricular myocytes of control (*black spectrum, left panel, n=4*) and atrophic (*blue spectrum, right panel, n=4*) hearts. At a pipette potential of −40 mV, the extracellular solution was replaced by a Li^+-based solution resulting in an outward current (I_{NCX}). **e** Mean current density of I_{NCX}. *Numbers in columns* represent the number of individual cells. *$*P<0.05$; $**P<0.01$; $***P<0.001$ (two-way ANOVA followed by Newman–Keuls post hoc test). *Endo* endocardial myocytes, *Epi* epicardial myocytes

The density of the Na^+–Ca^{2+} exchanger current (I_{NCX}) was quantified in further patch clamp experiments as previously described [26]. Using a K^+-free Tyrode solution, myocytes were held at $V_{Pip}=-40$ mV. Switching the extracellular solution to a Li^+-based bath solution activated I_{NCX} in its reverse mode, resulting in an outward current (Fig. 2d). I_{NCX} was quantified as the difference between the holding current and the maximal outward current. Using this approach, no significant difference was detected in the functional expression of

I_{NCX} (Fig. 2e), implicating that the Na^+–Ca^{2+} exchanger is not altered in early cardiac atrophy.

Diastolic Ca^{2+} release

An increased occurrence of spontaneous SR Ca^{2+} release could also contribute to the marked decrease in the amount of Ca^{2+} stored in the SR. We therefore quantified the frequency of Ca^{2+} sparks in isolated ventricular cardiac myocytes. Atrophic cardiac myocytes showed a significantly lower frequency of spontaneous diastolic Ca^{2+} sparks (∼−45 %; Fig. 3a). Also, the fraction of cells showing a very low Ca^{2+} spark activity (<0.5 sparks/100 μm/s) was twofold higher in the atrophic group (46 vs. 23 %; Fig. 3a). These results argue against a major contribution of an increased SR Ca^{2+} leak to the reduced SR Ca^{2+} content in the atrophic myocardium at this stage.

The occurrence of spontaneous Ca^{2+} release depends on the SR Ca^{2+} load [42]. Thus, the reduced spark frequency in atrophic hearts may be a consequence of the reduced SR Ca^{2+} load. To address this issue, we assessed the effect of a partial depletion of the SR on the Ca^{2+} spark frequency in control myocytes using thapsigargin as previously described [44]. To match the lower SR Ca^{2+} load of atrophic cardiac myocytes, control cardiac myocytes were treated with 100 nmol/l of the SERCA blocker thapsigargin. This resulted in a reduction of the caffeine-induced Ca^{2+} release from the SR by 54±12 % ($n=10$), which closely resembles the depletion in atrophic cardiac myocytes. Quantification of spontaneous Ca^{2+} release in control and thapsigargin-treated cardiac myocytes revealed that the partial depletion of the SR caused a ∼65 % lower mean Ca^{2+} spark frequency and a 2.5-fold higher fraction of cells with a very low spark activity (Fig. 3b). Thus, a quantitatively nearly identical reduction of the SR Ca^{2+} load was associated with a moderately smaller reduction of the spark frequency in atrophic (∼−45 %) than in control (∼−65 %) cardiac myocytes. Since this difference may be the consequence of an increased functional expression of RyRs, we determined their abundance and the phosphorylation (Fig. 3c–e). Indeed RyR expression was significantly increased (∼130 % of control; Fig. 3d) in the atrophic myocardium, whereas its phosphorylation at S^{2814} was unaltered (Fig. 3e).
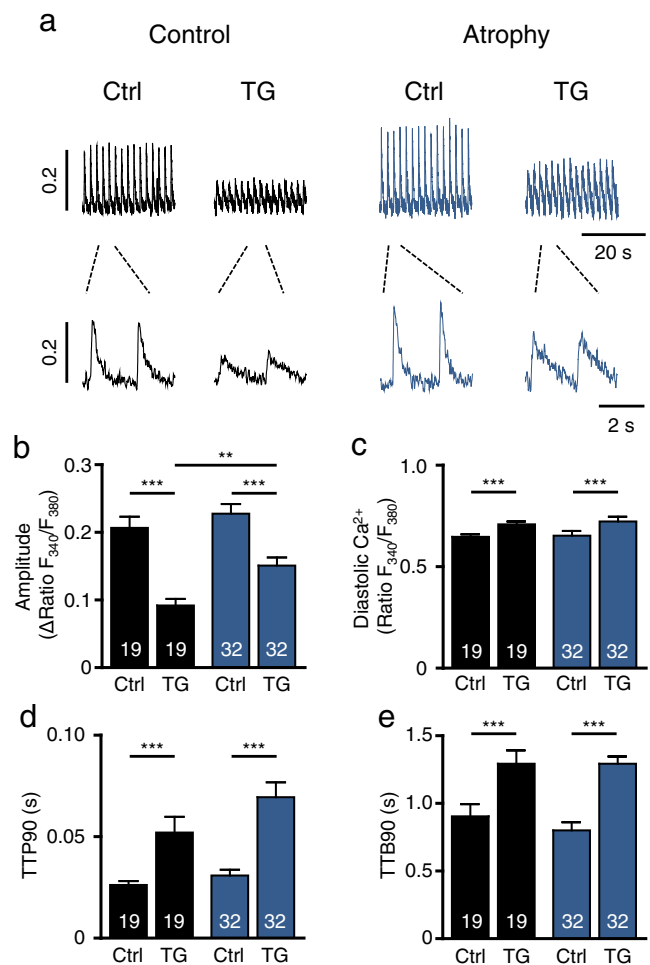
Systolic SR Ca^{2+} release

Unaffected Ca^{2+} transients in spite of a decreased SR Ca^{2+} content could be a consequence of an unaltered absolute amount of Ca^{2+} released from the SR during systole. To test this, the contribution of SR Ca^{2+} release to the total cytosolic Ca^{2+} transient was quantified. Ca^{2+} transients were assessed during continuous field stimulation in the absence and subsequently in the presence of thapsigargin (1 μmol/l; Fig. 4a). In the presence of 1 μmol/l thapsigargin, reuptake of cytosolic
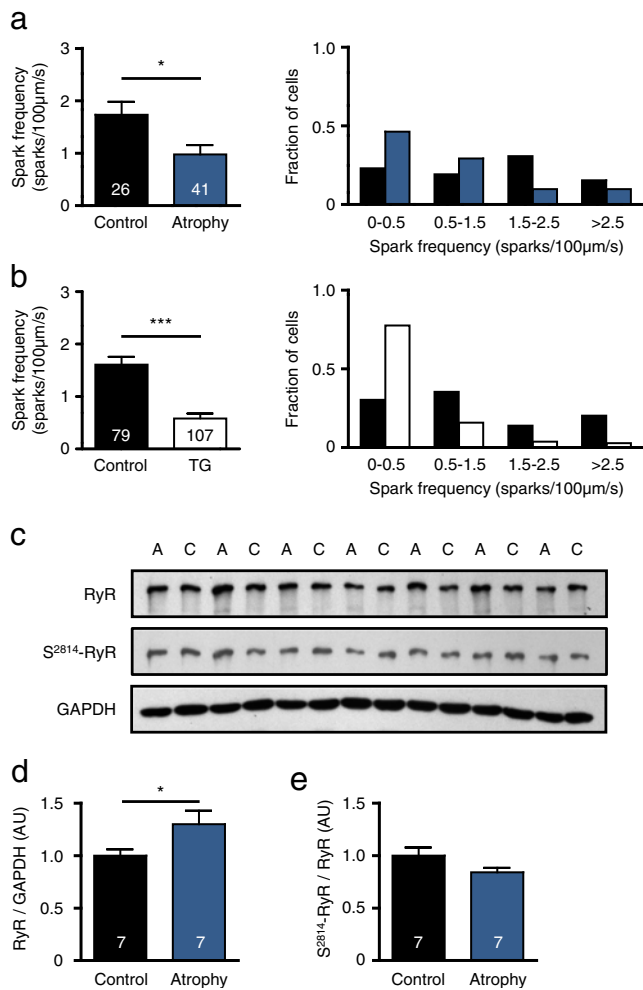
Fig. 3 Decreased Ca^{2+} spark frequency in myocytes of atrophic hearts and increased ryanodine receptor expression. **a** Average Ca^{2+} spark frequency (*left panel*) in left ventricular myocytes obtained from control (*black bar*, $n=7$) and atrophic (*blue bar*, $n=7$) hearts. The *right panel* illustrates the corresponding histogram. Ca^{2+} spark frequency was quantified over $1,856 \pm 75$ μm s per cell ($n=67$) following steady-state pacing. **b** Average Ca^{2+} spark frequency in left ventricular myocytes obtained from control hearts ($n=3$). Sparks were quantified in control myocytes (*black bar*) and in myocytes pre-treated with 100 nmol/l thapsigargin (*TG, white bar*). At this concentration, TG induced a depletion of the SR which closely matched the SR load of atrophic cardiac myocytes. The *left panel* illustrates the mean Ca^{2+} spark frequency and the *right panel* the corresponding histogram. **c** Representative immunoblots of RyR, S^{2814}-RyR and GAPDH in tissue samples of control (*C*) and atrophic (*A*) left ventricular myocardium. **d, e** Densitometric analysis of gene expression. RyR expression was normalised to GAPDH, and the phosphorylation of RyR at S^{2814} was normalised to total RyR amount in control (*black bar*) and atrophic (*blue bar*) tissue samples, respectively. All data are given relative to the mean of the expression in control hearts. *Numbers in columns* represent the number of cardiac myocytes (**a, b**) or tissue samples (**d, e**). *$*P<0.05$; $***P<0.001$ (Student's *t*-test)

Fig. 4 Decreased systolic Ca^{2+} release from the SR in myocytes of atrophic hearts. **a** Representative recordings of Ca^{2+} transients from myocytes isolated from control (*black spectrum, left panel*, $n=4$) and atrophic (*blue spectrum, right panel*, $n=5$) hearts. Following steady-state recordings (*Ctrl*), thapsigargin (1 μmol/l) was washed in (*TG*). **b–e** Summary data for the main parameters of Ca^{2+} transients under control conditions (*Ctrl*) and in the presence of thapsigargin (*TG*). *Numbers in columns* represent the number of individual myocytes. *TTP90* time to 90 % peak, *TTB90* time to 90 % baseline. $**P<0.01$; $***P<0.001$ (repeated-measures two-way ANOVA followed by Newman–Keuls post hoc test)

Ca^{2+} into the SR is fully inhibited, and the increase in cytosolic Ca^{2+} concentration during the following APs mainly derives from AP-induced Ca^{2+} influx [23]. If the total amount

of Ca^{2+} released from the SR was similar in both groups, the Ca^{2+} transient should be depressed by a comparable degree in both groups. In control myocytes, thapsigargin significantly reduced the amplitude of Ca^{2+} transients by ~55 %. In atrophic myocytes, however, the amplitude was reduced by only ~35 % (Fig. 4b). Application of thapsigargin increased diastolic Ca^{2+} levels (Fig. 4c) and the time course of the Ca^{2+} transients (Fig. 4d, e) similarly in myocytes of both groups. While the experimental condition of completely absent Ca^{2+} release from the SR is likely to interfere with Ca^{2+}-dependent LTCC inactivation and therefore possibly alters their contribution to the Ca^{2+} transient, these results suggest a reduced amount of Ca^{2+} released from the SR in the atrophic cardiac

Fig. 5 Increased Ca^{2+} influx during the AP in myocytes from atrophic hearts. **a** Representative APs and corresponding Cd^{2+} sensitive currents (I_{Ca}) recorded in endocardial and epicardial myocytes isolated from control (*black spectrum*, $n=3$) and atrophic (*blue spectrum*, $n=4$) hearts. The AP waveform (*upper panels*) of each individual cell was used to record the AP-driven Ca^{2+} influx via L-type Ca^{2+} channels (I_{Ca}, *lower panels*) of each myocyte in the voltage clamp configuration (AP clamp). I_{Ca} was calculated as the difference between the whole-cell current evoked by the AP waveform in the absence and in the presence of 30 µmol/l Cd^{2+}. **b, c** Mean values of AP duration (APD90, **b**) and of Ca^{2+} influx (Q_{Ca}, **c**). Q_{Ca} was calculated as the area under the curve of I_{Ca} normalised to the cell capacitance. **d** Representative immunoblots of Cav1.2 and calsequestrin (*CSQ*) of tissue samples from control and atrophic left ventricular myocardium. **e** Mean values of Cav1.2 expression normalised to CSQ in control (*black bar*) and atrophic (*blue bar*) tissue samples, respectively. All data are given relative to the mean of the expression in control hearts. *Numbers in columns* represent the number of individual myocytes (**b, c**) or tissue samples (**e**). *Endo* endocardial myocytes, *Epi* epicardial myocytes. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (ANOVA followed by Newman–Keuls post hoc test or Student's *t*-test)

myocytes during systole. Thus, the Ca^{2+} transient in atrophic cardiac myocytes must be maintained by a compensatory mechanism other than an increased fractional release from the SR.

Systolic Ca^{2+} influx

A possible explanation for unchanged Ca^{2+} transients in the presence of a reduced SR Ca^{2+} load is an increased Ca^{2+} influx during the AP. Supporting this hypothesis, we have previously reported an increased AP duration in atrophic cardiac myocytes [40]. Ca^{2+} influx into the cell via the LTCC is regulated by several factors, including the AP waveform, the availability of LTCC and the negative feedback loop induced by the Ca^{2+}-dependent LTCC inactivation. This complex regulation makes prediction of the absolute amount of systolic Ca^{2+} influx during the AP difficult. We therefore assessed the AP-induced Ca^{2+} influx using the AP clamp technique. This allows the quantification of the Ca^{2+} influx in individual cardiac myocytes using their own AP waveform [51, 53]. The steady-state AP waveform of the cardiac myocyte under investigation was recorded in the current clamp mode at the beginning of each patch clamp experiment (Fig. 5a, upper panels). Consistent with our previous findings [40], cardiac atrophy was associated with significantly prolonged APs in endocardial myocytes (~150 % of control) and epicardial myocytes (~250 % of control; Fig. 5b). After switching to the voltage clamp mode, the membrane potential of each individual myocyte was clamped to its own previously recorded AP. The AP-induced current was recorded in the absence and presence of the Ca^{2+} channel blocker Cd^{2+} (30 µmol/l). Subtraction analysis of the resulting currents yielded the Cd^{2+}-sensitive current, which gives an estimate for the AP-induced Ca^{2+} current through LTCC (I_{Ca}; Fig. 5a,
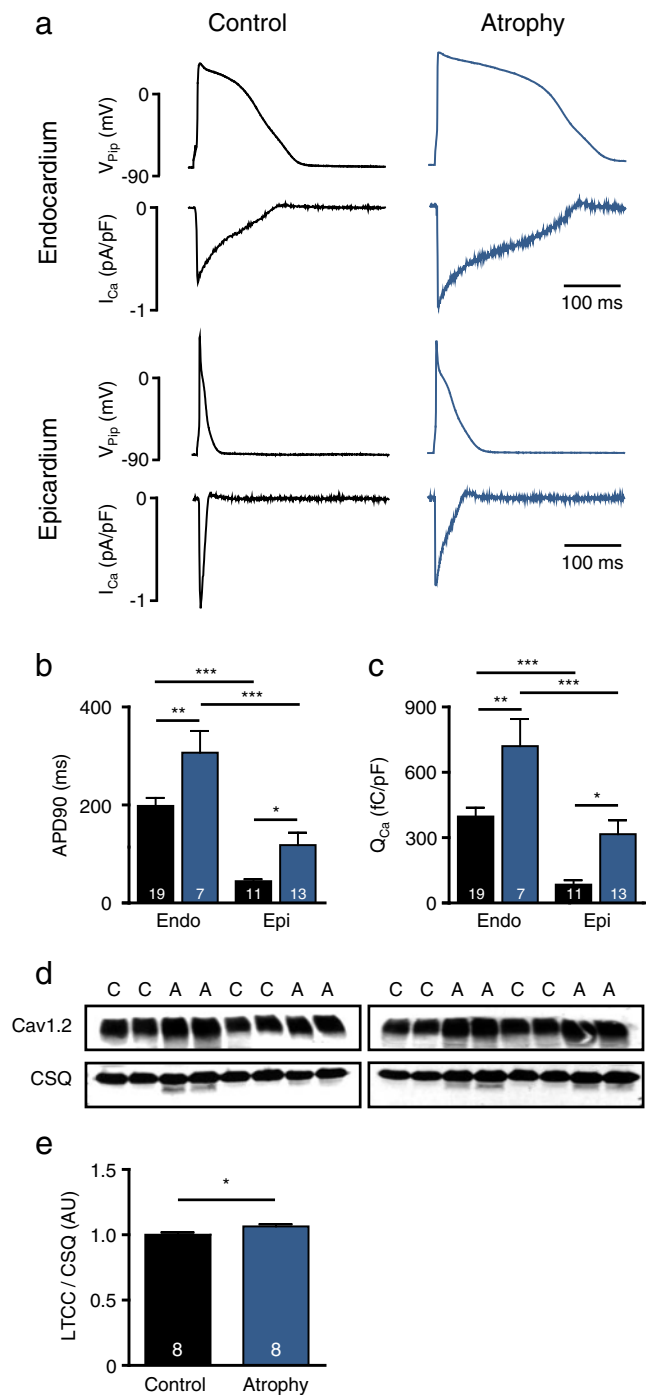
lower panels). To calculate total Ca^{2+} influx during the AP, I_{Ca} was integrated (Q_{Ca}). Q_{Ca} was approximately two- to fourfold higher in atrophic endocardial and epicardial myocytes than in control myocytes from the corresponding layer (Fig. 5c). In addition, Western blot analysis revealed an upregulation of Cav1.2 subunits in the atrophic myocardium (Fig. 5d, e). These results indicate that in atrophic cardiac myocytes Ca^{2+} influx through LTCC is augmented both by an increased

electrical driving force caused by prolonged APs and by an increased membrane abundance of Ca^{2+} channel protein.

Discussion

Two major interdependent mechanisms contribute to the systolic elevation of cytosolic Ca^{2+} in ventricular cardiac myocytes (Fig. 6a). Systolic Ca^{2+} cycling is initiated by the sarcolemmal Ca^{2+} influx through LTCC during the AP. Besides directly raising the intracellular Ca^{2+} concentration, this triggers further Ca^{2+} release from the SR (Ca^{2+}-induced Ca^{2+} release). The amount of Ca^{2+} released from the SR depends on the size of the trigger signal, on the SR Ca^{2+} load and on the Ca^{2+} release threshold [6, 13, 24, 50]. In cardiac myocytes, the majority of the total Ca^{2+} increase is normally met by Ca^{2+} release from the SR [4, 13]. Here we show that cardiac atrophy induces a pronounced alteration of the balance of Ca^{2+} cycling between the two major Ca^{2+} sources. While SR Ca^{2+} content and the amount of Ca^{2+} released during the Ca^{2+}-induced Ca^{2+} release are greatly reduced in atrophic hearts, this reduced contribution of the SR is fully compensated by an increased Ca^{2+} influx through LTCC. As a result, systolic Ca^{2+} transients remain normal in atrophic myocardium.

In myocytes of atrophic hearts, the amount of Ca^{2+} stored in the SR was ~55 % lower than in myocytes isolated from control hearts. This may result from an increased SR Ca^{2+} leak and/or a decreased Ca^{2+} uptake. Diastolic spark frequency was decreased by ~45 % in atrophic cardiac myocytes, which argues against a relevant contribution of an increased diastolic Ca^{2+} leak to the SR depletion. SR Ca^{2+} content itself, however, influences the spark frequency, complicating the interpretation. A quantitatively similar reduction of SR Ca^{2+} load induced by a pharmacological SERCA inhibition in control cardiac myocytes induced a modestly larger SR Ca^{2+} spark reduction (~−65 %). This finding suggests that the reduced frequency of spontaneous Ca^{2+} release in atrophic cardiac
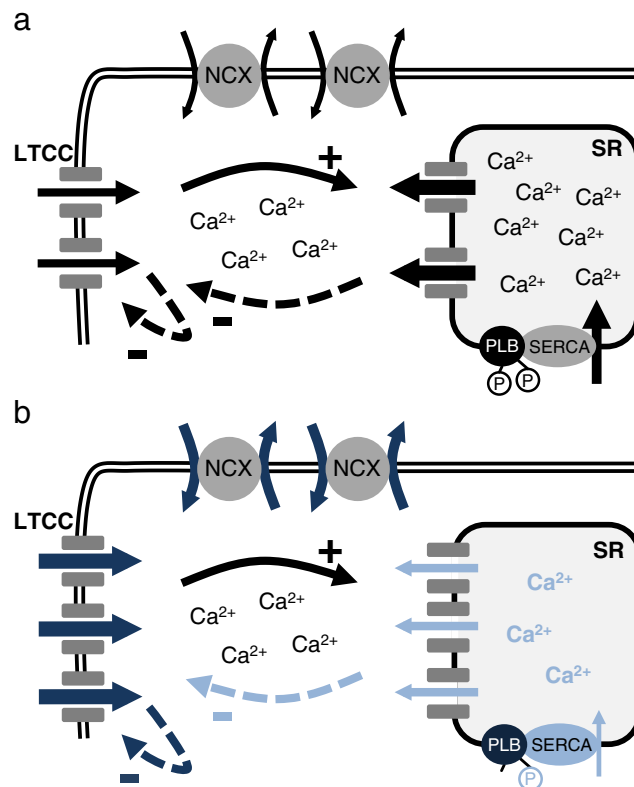
Fig. 6 Ca^{2+} cycling in normal and atrophic cardiac myocytes. Schemes illustrating Ca^{2+} cycling under steady-state conditions in normal (**a**) and in atrophic cardiac myocytes (**b**). **a** In normal ventricular cardiac myocytes, Ca^{2+} influx during the AP through LTCC triggers Ca^{2+} release from the SR. Increasing cytosolic Ca^{2+} concentrations (by Ca^{2+} influx and SR Ca^{2+} release) inhibit LTCC, thus limiting further Ca^{2+} influx. The cytosolic Ca^{2+} concentration is reduced by the reuptake of Ca^{2+} into the SR via SERCA and by extrusion via Na^+–Ca^{2+} exchanger. **b** In atrophic hearts, SERCA activity is reduced secondary to a diminished phosphorylation of PLB, resulting in reduced Ca^{2+} uptake into the SR, SR Ca^{2+} content and SR Ca^{2+} release during systole. Accordingly, the inhibition of LTCC by Ca^{2+} released from the SR is reduced. This, in combination with prolonged APs and an increased density of LTCC on the cell surface allows for a larger net Ca^{2+} influx during the AP. Relative changes compared to the control myocytes are indicated by colours (*dark blue* increase, *light blue* decrease). *LTCC* L-type Ca^{2+} channel, *NCX* Na^+–Ca^{2+} exchanger, *SR* sarcoplasmic reticulum, *PLB* phospholamban, *SERCA* SR Ca^{2+} ATPase

myocytes may be mainly a consequence of the reduced SR Ca^{2+} load which is partly offset by an increased ryanodine receptor density. Ca^{2+} uptake into the SR is governed by SERCA activity, which depends on the cytosolic Ca^{2+} concentration and modulation by several regulator proteins, including PLB and sarcolipin [25]. Under steady-state conditions, even a mild reduction in SERCA activity can lead over time to a markedly reduced SR Ca^{2+} load. Consistently, we have previously reported a decrease in phosphorylation of PLB in atrophic myocardium which is expected to contribute to a reduction in SERCA activity [41]. Given that the decay of the intracellular Ca^{2+} transient is unaltered in atrophic myocardium (Fig. 1) [22, 46], it seems likely that SERCA activity is reduced to a degree that the resulting slowing of the Ca^{2+} uptake into the SR is fully compensated for by Ca^{2+} transport via the Na^+–Ca^{2+} exchanger. Taken together, the data currently available indicate that the reduction in SR Ca^{2+} load is predominantly a consequence of a moderately decreased SERCA activity, possibly mediated by reduced PLB phosphorylation and potentially in combination with a reduced set point of SR Ca^{2+} content [24] or an increased nonspark diastolic Ca^{2+} release [39]. It cannot be excluded that during an earlier phase of atrophic remodelling, an increased SR leak contributes to the development of the reduced SR Ca^{2+} load.

Consistent with previous reports, no significant differences were detected regarding diastolic Ca^{2+}, systolic Ca^{2+} amplitude or the onset and decay of the Ca^{2+} transients [22, 46]. In principle, the Ca^{2+} transient in atrophic hearts could be conserved by an unaltered amount of Ca^{2+} released from the SR in spite of its depletion (i.e. higher fractional Ca^{2+} release). Using the SERCA blocker thapsigargin we could demonstrate that in atrophic myocytes the quantity of Ca^{2+} released from the SR was significantly lower than in control cardiac myocytes. Therefore, to maintain a normal Ca^{2+} transient, an increased Ca^{2+} influx through LTCC has to compensate for the depressed SR Ca^{2+} release. Ca^{2+} influx is mainly governed by the shape of the AP and the availability of Ca^{2+} channels. We have previously reported that a diminished density of the transient outward K^+ current (I_{to}) in combination with an increased density of the L-type Ca^{2+} current results in a net prolongation of APs in atrophic hearts [40]. We now demonstrate that this electrophysiological remodelling is associated with a two- to fourfold increase in Ca^{2+} influx during the AP, providing a mechanism for the maintenance of the systolic Ca^{2+} transient.

Sarcolemmal Ca^{2+} influx increases the intracellular Ca^{2+} concentration, hence activating ryanodine receptors and initiating the Ca^{2+}-induced Ca^{2+} release (Fig. 6a). At the same time, however, the increasing Ca^{2+} concentration in the subsarcolemmal space will limit further Ca^{2+} influx by inactivating LTCC [4]. This negative feedback mechanism regulates the relation between Ca^{2+} influx and Ca^{2+} release from the SR and assures a finely tuned Ca^{2+} transient [13]. In

cardiac atrophy, two remodelling processes (increased Ca^{2+} influx and decreased SR Ca^{2+} release) seem to exactly balance each other, resulting in unchanged Ca^{2+} transients (Fig. 6b). A primary decrease of SR Ca^{2+} load and SR Ca^{2+} release would lead to a slower increase of subsarcolemmal Ca^{2+} concentrations during systole, thus causing less inhibition of LTCC. This would facilitate escalating Ca^{2+} influxes until the initial Ca^{2+} transient is balanced again. If the initial response to cardiac atrophy was an augmentation of Ca^{2+} influx through LTCC, the resulting increase in cytosolic Ca^{2+} concentrations may induce a reduction of the SR Ca^{2+} load and SR Ca^{2+} release, e.g. via a diminished phosphorylation of PLB, eventually resulting in a new steady state. This functional coupling between Ca^{2+} influx and Ca^{2+} release from the SR seems to be supported by changes in the expression of ion channels which are relevant for regulating Ca^{2+} influx with an upregulation of LTCC (Fig. 5) and a downregulation of K^+ channel subunits underlying I_{to} [40]. The expression of all of these channels is modulated via the CaMKII-DREAM and/or calcineurin pathways by the Ca^{2+} concentration [32, 37]. Collectively, these observations suggest that during atrophic remodelling, localized changes of the intracellular Ca^{2+} concentration preserve the systolic Ca^{2+} transient both via a functional feedback as well as via a modulation of ion channel expression.

Cardiac hypertrophy induced by an increased afterload and cardiac atrophy have been proposed to induce a common pattern of cellular remodelling [2, 12, 36]. Consistent with this concept, we have previously shown that cardiac atrophy is associated with similar prolongations in cardiac action potentials as cardiac hypertrophy [3, 15, 31, 40, 52]. This may potentially create an arrhythmogenic substrate, e.g. by inducing early after depolarizations. Here, we demonstrate that the same changes in Ca^{2+} and K^+ channel expression underlying the AP prolongation are functionally also associated with an increased AP-induced Ca^{2+} influx in atrophic hearts. Accordingly, the concept of a common response pattern seems also to hold true for these aspects of cell physiology. In contrast, however, cardiac atrophy is associated with reduced PLB phosphorylation, a reduced SR Ca^{2+} content and a reduced SR Ca^{2+} release. Each of these aspects could be expected to be increased in cardiac hypertrophy [5, 9, 31, 47]. The remodelling of these SR-associated properties in cardiac atrophy therefore appears to be opposite to that in cardiac hypertrophy. The remodelling of the resulting systolic Ca^{2+} transients cannot be assigned to either concept. While cardiac hypertrophy is typically associated with an increased amplitude of systolic Ca^{2+} transients [9, 47], atrophic hearts have identical Ca^{2+} transients as control hearts. Thus, the load-dependent remodelling of systolic Ca^{2+} transients may follow a "rectification": while they are increased in response to an increased cardiac workload, they are conserved during states of decreased cardiac workload.

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