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Cardiomyocyte Ca^{2+} handling and structure is regulated by degree and duration of mechanical load variation

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Abstract

Cardiac transverse (t)-tubules are altered during disease and may be regulated by stretch-sensitive molecules. The relationship between variations in the degree and duration of load and t-tubule structure remains unknown, as well as its implications for local Ca^{2+}-induced Ca^{2+} release (CICR). Rat hearts were studied after 4 or 8 weeks of moderate mechanical unloading [using heterotopic abdominal heart–lung transplantation (HAHLT)] and 6 or 10 weeks of pressure overloading using thoracic aortic constriction. CICR, cell and t-tubule structure were assessed using confocal-microscopy, patch-clamping and scanning ion conductance microscopy. Moderate unloading was compared with severe unloading [using heart-only transplantation (HAHT)]. Mechanical unloading reduced cardiomyocyte volume in a time-dependent manner. Ca^{2+} release synchronicity was reduced at 8 weeks moderate unloading only. Ca^{2+} sparks increased in frequency and duration at 8 weeks of moderate unloading, which also induced t-tubule disorganization. Overloading increased cardiomyocyte volume and disrupted t-tubule morphology at 10 weeks but not 6 weeks. Moderate mechanical unloading for 4 weeks had milder effects compared with severe mechanical unloading (37% reduction in cell volume at 4 weeks compared to 56% reduction after severe mechanical unloading) and did not cause depression and delay of the Ca^{2+} transient, increased Ca^{2+} spark frequency or impaired t-tubule and cell surface structure. These data suggest that variations in chronic mechanical load influence local CICR and t-tubule structure in a time- and degree-dependent manner, and that physiological states of increased and reduced cell size, without pathological changes are possible.

Keywords: assist device • Ca^{2+} handling • excitation–contraction coupling

Introduction

Depolarization by the action potential initiates L-type Ca^{2+} channel (LTCC) opening, resulting in local Ca^{2+} influx. This influx triggers ryanodine receptor (RyR) opening and mass release of Ca^{2+} from the sarcoplasmic reticulum (SR) in a process known as Ca^{2+}-induced Ca^{2+} release (CICR). It is mainly the Ca^{2+} released from the SR which activates the myofilaments causing contraction. CICR is therefore a critical step in excitation–contraction coupling in ventricular myocytes. The efficiency by which the Ca^{2+} influx through LTCC activates the RyRs is determined by a number of factors, including the spatial proximity of LTCCs and RyRs [1]. An elaborate series of LTCC-rich membrane invaginations, the transverse (t)-tubules, optimize CICR by promoting structural interaction of LTCC around RyR clusters [2, 3]. Furthermore, on a whole cell level, the t-tubules allow the rapid delivery of the excitation depolarization in close proximity to RyR clusters throughout the cell depth, promoting synchronous SR Ca^{2+} release and contraction.

T-tubule dysfunction is a major feature of heart failure in human cardiomyocyes [4], and in animal models of overload, myocardial infarction and arrhythmia (Reviewed in [3]). Mechanically overloaded hearts show disruption of the t-tubule system [5]. Mechanical unloading can also impact on the t-tubules and local CICR, as well as cause contractile dysfunction [6, 7]. Mechanical unloading can promote reverse functional and ultrastructural remodelling in heart failure [8, 9], but these improvements regress and cause further dysfunction with prolonged unloading [9]. These studies suggest that chronic load variation may have dynamic effects on the myocardium, which may be dependent on their degree and duration.

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Variations in load are clinically relevant as prolonged mechanical overload (*e.g.* in aortic stenosis) results in heart failure, and unloading using left ventricular assist devices (LVAD) is used to treat patients with heart failure. LVADs have shown promise in reversing pathological remodelling caused by heart failure [10]. However, initial functional improvements during LVAD therapy regress with prolonged mechanical unloading [11]. Reducing the degree of unloading could prevent these long-term negative effects and possibly favour a stable myocardial recovery, but this has not been thoroughly investigated [12, 13]. Defining the cellular response to variations in degree and duration of mechanical load is an important element of developing a fuller understanding of the mechanisms of these diseases and therapies.

The principle that prolonged mechanical overloading or unloading disrupt t-tubule structure is established (reviewed in [3]). However, whether there is an optimal range of degrees and durations of load variation, and the transition to a non-physiological range is not clear. In this study, we hypothesized that different degrees and durations of mechanical load differentially impact on t-tubule structure and local CICR. To address this, we studied cardiomyocyte t-tubule and surface structure from hearts undergoing chronic mechanical unloading and overloading for different durations.

Materials and methods

Syngeneic male Lewis rats (200–300 g) were used for all experiments, and all procedures followed Home Office regulations and Directive 2010/63/EU. Buphrenorphine at 0.05 mg/k was given at six hourly intervals throughout the post-operative recovery up to a period of 2 days following operation. Local and Funding agency ethical review was granted.

Animal models

Mechanical unloading

Moderate mechanical unloading *(UN)* was obtained by transplanting a heart–lung block from a donor animal into the abdomen of a syngeneic recipient [12]. Briefly, the ascending aorta of the donor was anastomosed to the recipient abdominal aorta. Coronary blood flow is directed to the right heart *via* the coronary sinus, through the pulmonary circulation and then to the LV. Therefore, the LV only ejects the coronary and not the systemic return, and is moderately mechanically unloaded. *Severe*, full unloading *(S-UN)* [7, 14, 15] was obtained using the heterotopic abdominal heart-only transplantation which abolishes blood return *via* the pulmonary veins completely. We have examined the effects of this technique on t-tubule structure in a previously published study [6] and have included some of the data here for comparison (Table 1 only). The

Table 1 Severe but not moderate mechanical unloading is associated with pathological remodelling of the t-tubule system

	UN 4	S-UN 4	*P* value
Ratio units			
Cell volume	0.63 ± 0.04 (n = 59)	0.43 ± 0.02 (n = 90)	***
T-tubule density	0.89 ± 0.03 (n = 50)	1.03 ± 0.02 (n = 90)	***
T-tubule regularity	1.10 ± 0.08 (n = 53)	0.13 ± 0.01 (n = 49)	***
Z-groove index	0.93 ± 0.15 (n = 6)	0.47 ± 0.03 (n = 17)	0.064
Ca^{2+} transient TTP	1.12 ± 0.05 (n = 46)	1.35 ± 0.08 (n = 42)	*
Ca^{2+} transient amplitude	0.99 ± 0.04 (n = 46)	0.71 ± 0.04 (n = 42)	***
Ca^{2+} transient T50	1.24 ± 0.03 (n = 46)	1.66 ± 0.12 (n = 42)	**
Ca^{2+} transient T90	1.24 ± 0.02 (n = 46)	1.36 ± 0.07 (n = 42)	0.997
Ca^{2+} transient VTTP	1.15 ± 0.07 (n = 46)	1.80 ± 0.16 (n = 42)	**
$I_{Ca,L}$ peak current	0.77 ± 0.06 (n = 26)	1.04 ± 0.04 (n = 15)	**
Spark frequency	1.18 ± 0.17 (n = 73)	3.85 ± 0.65 (n = 87)	***
Spark peak	1.12 ± 0.02 (n = 297)	0.96 ± 0.01 (n = 459)	***
Spark half-width	1.03 ± 0.02 (n = 242)	1.23 ± 0.02 (n = 410)	***
Spark duration	1.23 ± 0.04 (n = 242)	1.74 ± 0.06 (n = 410)	***

Table shows parameters of local CICR in 4-week moderately unloaded (UN 4) and severely unloaded cardiomyocytes (S-UN 4) as a proportion of control values. *N* numbers are given in brackets. Values from S-UN 4 group were derived from [6]. Values in ratio units. As the respective controls did not differ significantly from one another and to allow rigorous comparison of the effect of moderate and severe unloading, values have been normalized to their controls.
* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

recipient's native heart acted as a control in these experiments. Although cardiac work of the unloaded heart has not been quantified in this study, multiple studies show that the unloaded heart shows atrophy as a result of reduced pre-load (reviewed in [16]).

Thoracic aortic constriction (TAC) model

The TAC model was used to overload the heart by increasing afterload. A right thoracotomy was made, and the ascending aorta exposed. A suture was looped around the aorta and tied over a 0.9 mm bar placed between the ascending aorta and the suture. The suture was tied tightly and the needle removed. Sham-operated rat hearts acted as a control [17].

Duration of mechanical load variation and cell isolation

UN hearts and the normal recipient were harvested after 4 or 8 weeks. Mechanically overloaded hearts were harvested after 6 or 10 weeks.

Single LV cardiomyocytes were isolated as described previously [18]. All experiments were conducted at 37°C.

T-tubule imaging

Cells were suspended in buffer containing (in mM) 120 NaCl, 5.4 KCl, 5 $MgSO_4$, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES (free acid) and 0.2 $CaCl_2$; pH 7.4, and loaded for 10 min. with 10 μM of di-8-ANEPPS. Rod-shaped cardiomyocytes were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) through a Zeiss EC Plan-NeoFluar x40 oil-immersion lens [6]. Di-8-ANEPPS (and Fluo-4) was excited using the 488-nm line of an argon laser, and the emitted fluorescence was collected through a 505-nm long-pass filter.

High-resolution images of the cells were taken in a plane where t-tubules were visible to study their structure. Images at all cell planes were taken at a lower resolution and analysed using a custom-written macro in ImageJ (http://rsb.info.nih.gov/ij/) where t-tubule density and cell volume were calculated. The high-resolution images were adapted into binary images in ImageJ, and t-tubule plot profiles of a central portion (of fixed dimensions) of these images were generated, as previously described [6]. Fourier analysis of the plot profiles was performed with a custom-written script in MATLAB R2006b (The MathWorks, Inc., Natick, MA, USA). Importantly, this analysis was performed by an investigator blinded to the identity of the groups. A confocal plane devoid of nuclei was selected, near the middle of the cell. The most central, representative section was chosen.

Ca^{2+} spark and transient imaging

To measure local Ca^{2+} changes (sparks and transients), the cells were loaded with 10 μM Fluo-4 AM for 20 min. The cells were superfused with normal Tyrode's solution (NT) (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with 2 M NaOH) and imaged using the confocal microscope described above. Cells were field stimulated at 1 Hz to obtain steady-state contraction. After a 30-sec. period of rest, line scans were collected and analysed using custom-written algorithms in MATLAB. Ca^{2+} spark detection criteria were set at 3.8 S.D. above the background noise and Ca^{2+} spark amplitude was classified as peak fluorescence over background fluorescence (F/Fo), as previously described (7). Ca^{2+} spark frequency, width at half-maximum (fWhm) and duration at half-maximum (fDhm) were also measured.

Ca^{2+} transients were studied by field stimulating the cells at 1 Hz and recording line scans. Amplitude, time-to-peak (TTP), variance in time-to-peak (VTTP) and time to 50% (T50) and 90% (T90) decline in the transients were measured using MATLAB [6].

Measurement of SR Ca^{2+} content

Cardiomyocytes were loaded with 10 μM Indo-1 AM (Molecular Probes, Eugene, OR, USA) for 30 min. at room temperature. The assessment of SR Ca^{2+} content was achieved by 1 Hz stimulation to steady-state contraction upon which stimulation was stopped, followed by the rapid application of 20 mM caffeine in Na^+/Ca^{2+} free solution [(in mM): 140 LiCl, 6 KOH, 1 $MgCl_2$, 10 glucose, 10 HEPES, 0.1 EGTA; pH to 7.4 with 1 M LiOH] for 5 sec. Peak amplitude of caffeine-induced Indo-1 transient was taken as an index of SR Ca^{2+} content, and reported in Indo-1 (405/485) ratio units.

L-type Ca^{2+} current (ICa,L) recordings

ICa,L was measured in voltage-clamp mode as described previously [14]. The pipette resistance was ~2–3 MΩ, and the pipette-filling solution contained (in mM) 115 cesium aspartate, 20 tetraethylammonium chloride, 10 EGTA, 10 HEPES and 5 MgATP, pH 7.2. The external solution contained (in mM) 140 NaCl, 10 glucose, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ and 6 CsCl, pH 7.4. Current–voltage relationships for ICa,L were built using 450-ms depolarization steps from a holding potential of −40 mV (range −40 to +40 mV, in 5-mV increments) at 1 Hz. Then 200 μM Cd^{2+} was applied, and the protocol was repeated. Subtracted currents obtained were normalized to cell capacitance. All experiments were conducted at 37°C.

Scanning ion conductance microscopy (SICM)

The SICM setup has been described previously [4, 19]. We used hopping mode ion conductance microscopy without continuous feedback [20]. We obtained high-resolution images of the surface of freshly isolated LV cardiomyocytes from either control or unloaded hearts. In all SICM experiments, micropipettes and the bath solution contained the same physiological L-15 medium (Life Technologies, Inc., Parsley, UK), so that salt concentration gradient potentials and liquid junction potentials were not generated.

The z grooves are fine ultrastructural elements of the cell surface that have been linked to both regularity of t-tubules and their function constituents [19], but also signalling domains [21]. To quantify the data obtained during scanning, we introduced an index of the completeness of the Z grooves on the surface of cardiomyocytes (Z-groove index) [22].

Statistical analysis

Statistical analysis was performed with Prism4 software (GraphPad software Inc., San Diego, CA, USA), and the non-parametric Kruskal–Wallis test was used to assess statistical differences. Dunn's *post hoc* test was used to compare groups. Data are represented as mean ± standard error of the mean. A minimum of 3 rats were used in each experimental group and n numbers represent the number of cells studied, unless otherwise specified. * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$. As no statistical differences were detected between sham-operated 'TAC' controls and native recipient heart controls, these two groups were pooled in a single control group for clarity.

Results

Chronic mechanical load variation alters LV weight and cardiomyocyte volume

Four weeks UN significantly reduced cardiomyocyte volume by 25.7%, but did not cause a reduced left ventricular weight (Fig. 1). After 8 weeks UN, cell volume was further reduced (a 60.1% reduction compared with control, Fig. 1), as was LV weight. Mechanical overload for 6 weeks caused significant LV but not cardiomyocyte hypertrophy (Fig. 1). Mechanical overload for 10 weeks caused significant LV and cardiomyocyte hypertrophy (23.6% increase in cardiomyocyte volume compared with sham, Fig. 1).

The Ca^{2+} transient is affected by degree and duration of mechanical load

Four weeks UN did not influence the TTP of the Ca^{2+} transient, which was prolonged by 17.4% at 8 weeks unloading (Fig. 2). Four weeks UN did not affect the amplitude of the Ca^{2+} transient, but 8 weeks significantly reduced this (Fig. 2). Both 4 and 8 weeks UN increased the T50 and T90 decline in the Ca^{2+} transient. The VTTP, a measure of the dyssynchrony of the Ca^{2+} transient, was increased after 8 weeks but not 4 weeks UN (Fig. 2). TTP was unaffected by the duration of mechanical overload used in this study (Fig. 2). After 6 and 10 weeks of mechanical overload the amplitude of the Ca^{2+} transient was larger and the decline in the Ca^{2+} transient was faster compared with control. Six weeks of mechanical overload reduced VTTP of the Ca^{2+} transient, which was then increased to sham levels at 10 weeks (Fig. 2). SR Ca^{2+} content was augmented at 10 weeks of mechanical overload compared with control (Control: 0.39 ± 0.1 ratio units, $n = 30$ *versus* TAC 10 weeks: 0.47 ± 0.1 ratio units, $n = 29$, $P < 0.0001$). In summary, we found that UN progressively impaired whole cell Ca^{2+} cycling but that the two overloading conditions induced 'supernormal' responses, which resembled adaptation to physiological stress.

Ca^{2+} spark frequency and morphology is affected by degree and duration of mechanical load

Eight but not 4 weeks UN increased Ca^{2+} spark frequency. Four weeks UN increased the Ca^{2+} spark peak, which was reduced at 8 weeks UN compared with control (Fig. 3). Four and 8 weeks UN increased Ca^{2+} spark width and duration. Ten but not 6 weeks of mechanical overload increased Ca^{2+} spark frequency (Fig. 3). Six and ten weeks of mechanical overload enhanced Ca^{2+} spark peak and width. Six weeks of mechanical overload reduced Ca^{2+} spark duration, which was increased at 10 weeks compared with control. This suggests that Ca^{2+} sparks are affected by the direction, degree and duration of chronic changes in mechanical load.

T-tubule structure is sensitive to degree and duration of mechanical load

Neither 4 nor 8 weeks UN altered the t-tubule density (Fig. 4). Eight but not 4 weeks UN reduced the t-tubule regularity, measured as the

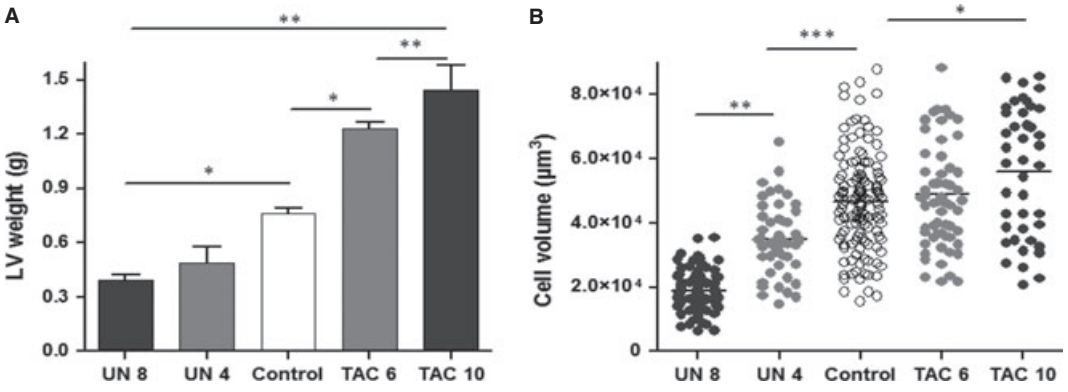

Fig. 1 Chronic mechanical load variation alters LV weight and cardiomyocyte volume. (A) LV weight of moderately unloaded and overloaded hearts (UN 8 $n = 3$, UN 4 $n = 4$, Control $n = 7$, TAC 6 $n = 5$, TAC 10 $n = 6$); (B) Cell volume of di-8-anepps stained LV cardiomyocytes from hearts subjected to chronic mechanical load variation in different periods (UN 8 $n = 80$, UN 4 $n = 50$, Control $n = 136$, TAC 6 $n = 60$, TAC 10 $n = 42$).

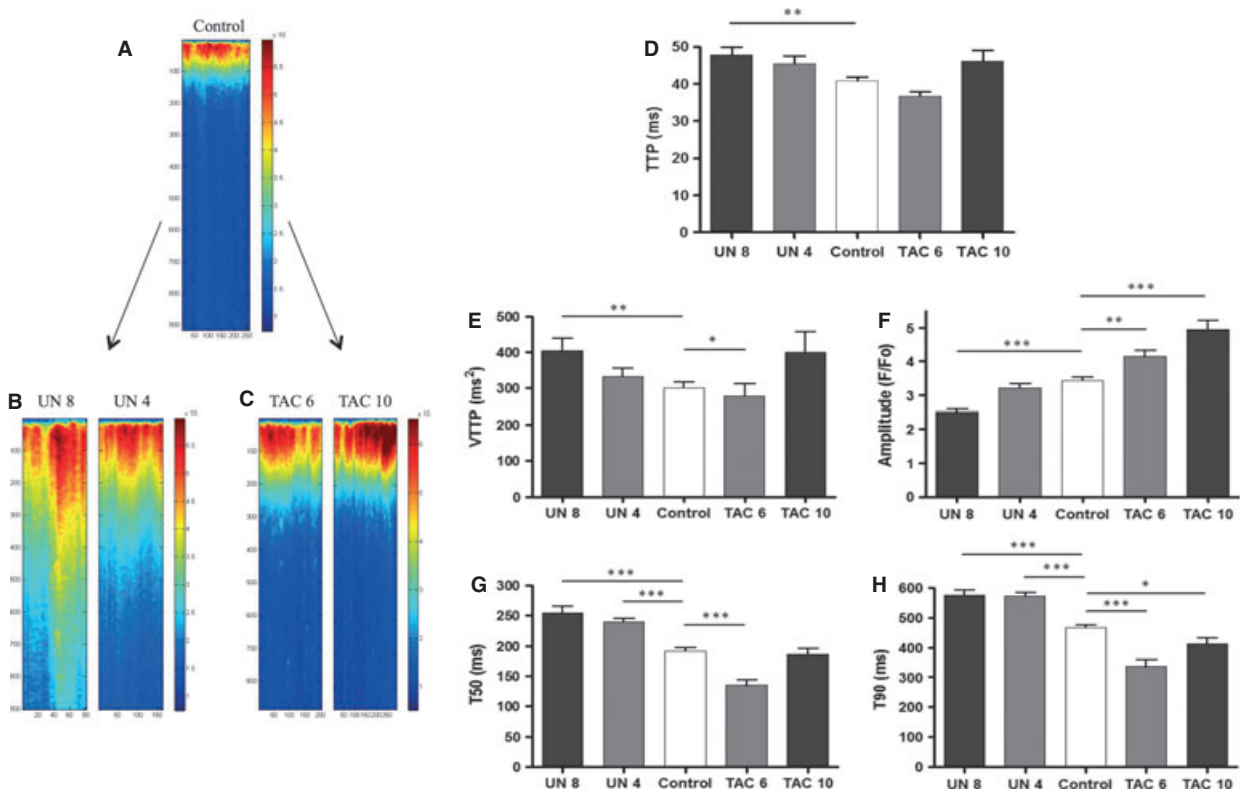

Fig. 2 The Ca^{2+} transient is affected by degree and duration of mechanical load. (**A**) Representative traces of stimulated Ca^{2+} transients taken during line scanning of control, (**B**) unloaded and (**C**) overloaded cardiomyocytes. (**D**) The time-to-peak of the Ca^{2+} transient. Graphs additionally show (**E**) Variance of the time-to-peak of the Ca^{2+} transient (a measure of the synchronicity of Ca^{2+} release throughout the cell) was measured as an index of the synchrony of Ca^{2+} release, (**F**) Amplitude, (**G**) time to 50% decline and (**H**) time to 90% decline was assessed. Values on the vertical aspect of line scans represent microseconds, and on the horizontal scale represent pixels. (UN 8 $n = 50$, UN 4 $n = 46$, Control $n = 141$, TAC 6 $n = 34$, TAC 10 $n = 21$).

peak of the Fourier transform. At 6 weeks of mechanical overload, neither t-tubule density nor regularity were affected (Fig. 4). However, 10 weeks of mechanical overload depressed both t-tubule density and regularity. These data show that t-tubule structure is progressively disrupted by either chronic unloading or overloading of the myocardium.

Severe but not moderate mechanical unloading at 4 weeks is associated with pathological remodelling of local CICR, t-tubule and cell structure

To examine the impact of moderate *versus* severe mechanical unloading we compared the results obtained after 4 weeks UN (as used throughout above) with results obtained using a model of severe unloading (S-UN), previously described and published [6]. We found that S-UN was associated with significantly smaller cell volume than UN (Table 1). Neither severe nor moderate unloading affected the t-tubule density with respect to control, although S-UN was surprisingly associated with higher t-tubule density compared with UN. While 4 weeks of UN did not alter t-tubule regularity, S-UN was associated with significant loss of regularity of the t-tubule system (Table 1).

S-UN increased the variance, prolonged the mean of the time-to-peak, time to 50% decline and reduced the amplitude of the Ca^{2+} transient compared with S-UN (Table 1). The ICa,L was unaffected by either form of mechanical unloading compared with control, but UN was associated with lower peak ICa,L, possibly due to the minor drop in t-tubule density (raw data as well as peak currents (at +5 mV) normalized to control are shown). The Ca^{2+} spark frequency, width and duration were increased by S-UN compared with UN (Table 1). Ca^{2+} Spark peak amplitude was reduced by S-UN.

To assess the impact of different degrees of unloading on the cell surface, we used scanning ion conductance microscopy. Normal cardiomyocytes are associated with fine undulations (z grooves), which contain the t-tubule openings. S-UN appeared to induce some changes to the cell surface but this effect was not significantly different to the effect of UN (Table 1). In summary, these experiments show that the effects on CICR and t-tubules are graded by the severity of mechanical unloading.

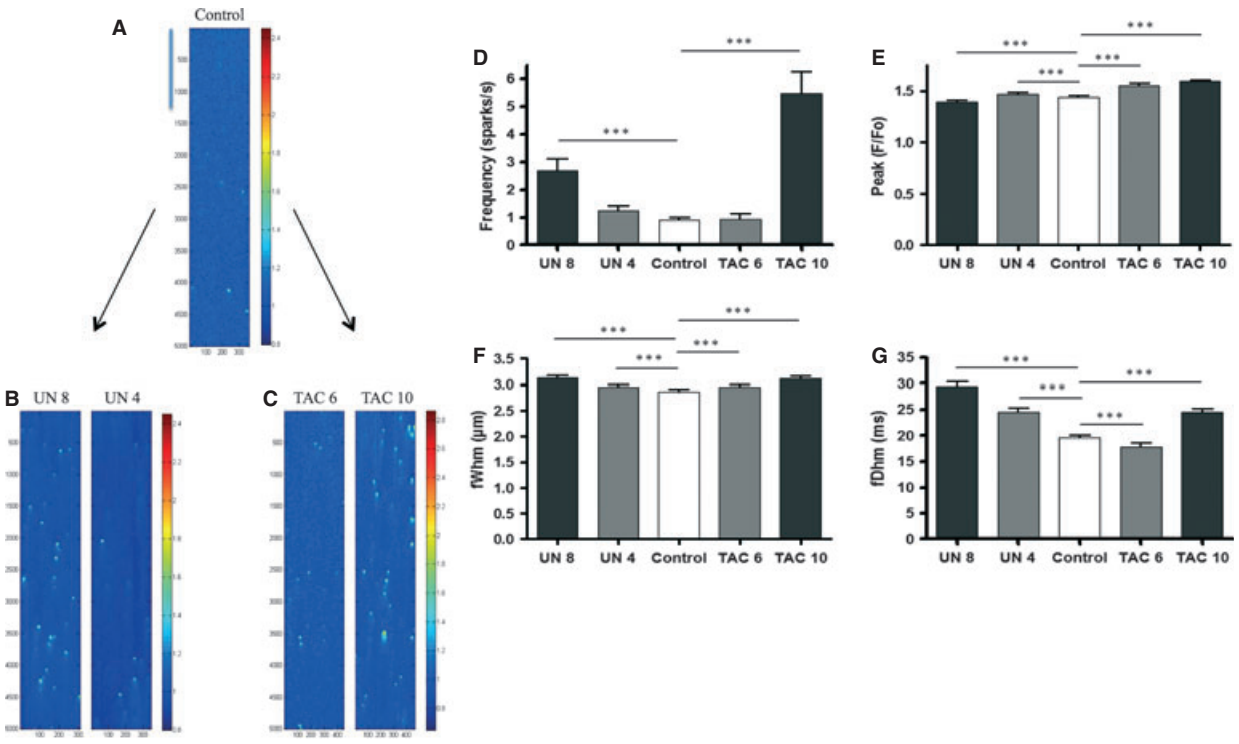

Fig. 3 Ca^{2+} spark frequency and morphology is affected by degree and duration of mechanical load. (**A**) Representative traces of quiescent cardiomyocytes taken in line-scan mode to measure Ca^{2+} spark features in control, (**B**) unloaded and (**C**) overloaded cardiomyocytes. Graphs show (**D**) Ca^{2+} spark frequency, (**E**) peak, (**F**) half-width and (**G**) duration. The vertical line alongside the control panel represents 1 sec. The horizontal numbers represent pixels. (Cells: UN 8 $n = 75$, UN 4 $n = 73$, Control $n = 173$, TAC 6 $n = 50$, TAC 10 $n = 28$ and sparks: UN 8 $n = 359$, UN 4 $n = 242$, Control $n = 447$, TAC 6 $n = 152$, TAC 10 $n = 513$).

Discussion

Our results shows that either severe chronic increases or decreases in load are associated with significant changes in local CICR and t-tubule structure of normal LV myocytes, whereas there were limited effects on these parameters by changes in load that are less severe or maintained for a shorter time period.

Effect of the degree of mechanical unloading

The differential impact of the two models for severe and moderate unloading has been corroborated in previous studies [13]. The observations by us and others that reduction in cell size obtained with moderate/short periods of unloading is accompanied by preserved cell function support the notion of 'physiological *hypo*trophy', similar, but opposite in direction, to physiological hypertrophy (Fig. 5). This should be distinct from 'pathological atrophy' which is observed after prolonged and severe unloading and is associated with dysfunction. Mechanical unloading using LV assist devices can produce major reverse remodelling and promote cardiac recovery [23], but prolonged unloading-induced atrophy may be a major impediment. One possible approach to prevent atrophic remodelling of the LV is the use of moderate mechanical unloading [12]. The data presented in this study supports the concept that pathological atrophic remodelling is delayed with the use of moderate mechanical assistance. CICR remodelling is a central defect in failing cardiomyocytes and reversal of these changes is specifically associated with cardiac recovery [24]. However, prolonged mechanical unloading can impair CICR [6].

Alterations in cell volume and myocardial hypertrophy

Both mechanical overload and unloading resulted in changes to the cell volume and LV weight. LV weight and cardiomyocyte volume did not necessarily change together, with a significant reduction in cell volume but not LV weight at 4 weeks of mechanical unloading, and a significant increase in LV weight but not cell volume at 6 weeks of mechanical unloading (Fig. 1). The mechanisms of this are unclear. The myocardial atrophy which results from mechanical unloading occurs despite enhanced Endothlin and Angiotensin II levels [25]. This may influence CICR independently, but this is beyond the scope of this study.

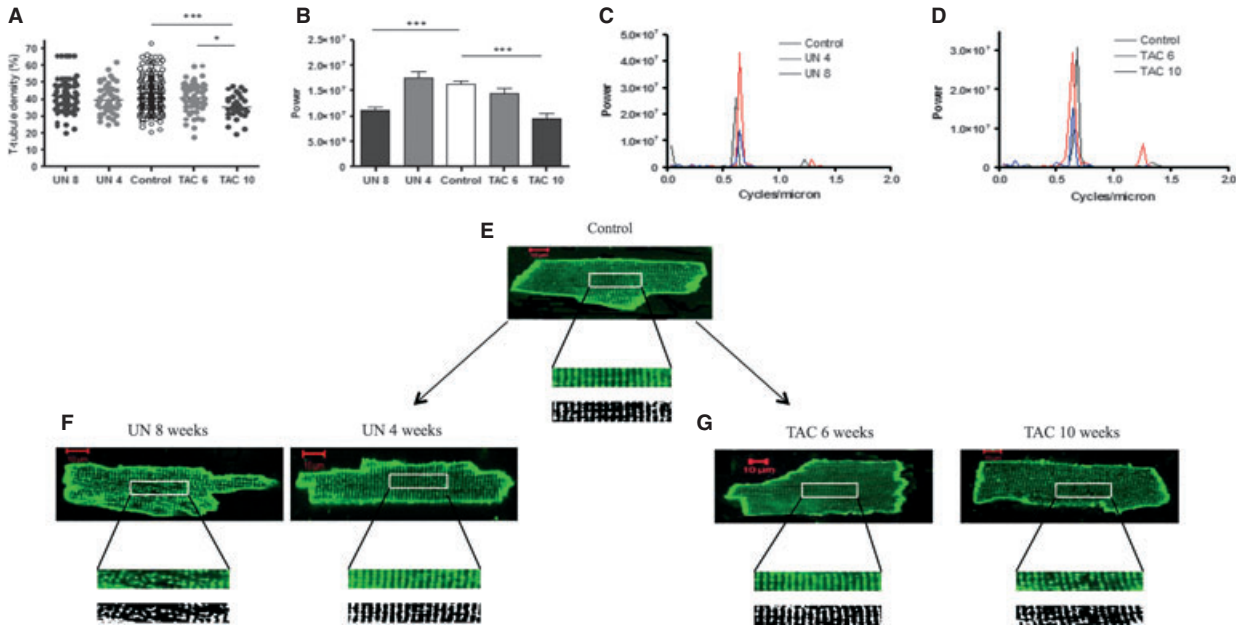

Fig. 4 T-tubule structure is sensitive to degree and duration of mechanical load. (**A**) T-tubule density was measured in cardiomyocytes from hearts undergoing chronic load variation. (UN 8 $n = 80$, UN 4 $n = 50$, Control $n = 157$, TAC 6 $n = 61$, TAC 10 $n = 31$) (**B**) T-tubule regularity was recorded using Fourier transforms of (**C**) unloaded and (**D**) overloaded cardiomyocytes (UN 8 $n = 82$, UN 4 $n = 53$, Control $n = 151$, TAC 6 $n = 42$, TAC 10 $n = 33$). (**E**) Representative images of di-8-anepps stained LV cardiomyocytes from control, (**F**) unloaded and (**G**) overloaded hearts. Inset shows binary images of central portions of the myocytes.

Load variation and local CICR

The Ca^{2+} transient also undergoes pathological remodelling in mechanical unloading, and was depressed in amplitude and delayed in its time course at 8 weeks of mechanical unloading. Shorter term unloading did not result in such changes. Severe mechanical unloading resulted in significantly more Ca^{2+} transient remodelling than moderate unloading, indicating a graded effect of degree of unloading. Mechanical overload resulted in augmented Ca^{2+} transient amplitude and faster decline at 6 and 10 weeks. These changes are likely to be a part of the physiological compensation to mechanical overload, and it is known that such physiological responses can be maintained up to 24 weeks after the institution of chronic mechanical overload [17]. Heart failure and the maladaptive response are

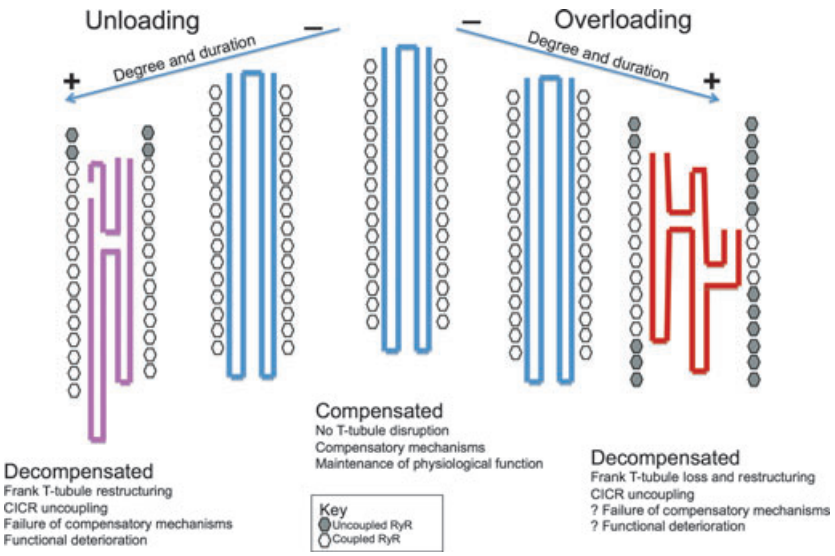

Fig. 5 Mechanical load variation regulates t-tubule structure. The figure illustrates the impact of variations in the degree and duration of mechanical overload and unloading on the t-tubule structure. Hexagons represent RyR units (shaded when uncoupled). When normal t-tubule structure permits close apposition of the L-type Ca^{2+} channel with RyR clusters, there is efficient CICR. However, during chronic or profound mechanical load variation, t-tubule restructuring or loss 'orphans' RyR clusters, causing dysfunctional CICR.

heralded by abnormal cellular structure and function. VTTP was increased after 8 weeks of mechanical unloading, indicating greater CICR dyssynchrony, related to the disruption of the t-tubule structure. Interestingly, we observed reduced VTTP (better synchrony) at 6 weeks of mechanical overload, possibly indicating enhanced cellular activation due to the augmented Ca^{2+} transient. This effect was lost at 10 weeks of mechanical overload, which accompanied t-tubule loss and disorganization. Whereas shorter term mechanical overload is within a physiological range and associated with preserved t-tubule structure, as in physiological hypertrophy of exercise [26], prolonged overload likely represents the beginning of a maladaptive response. The enhanced SR Ca^{2+} content observed at 10 weeks mechanical overload could account for the fact that despite the loss of the t-tubule network, relatively normal Ca^{2+} transients were observed. The increased SR Ca^{2+} content could mitigate the reduced ICa,L-RyR coupling, allowing an early stage of compensated T-tubule dysfunction. ICa,L was unchanged compared with control in both moderate and severe unloading. We suggest that the significant difference in ICa,L between normalized moderate and severe unloading (Table 1) appears to be functionally anomalous. Although we did not record the L-type Ca^{2+} current density in cells from hearts following TAC, previous studies indicate the L-type Ca^{2+} current density is unaffected by mechanical overload [27]. Multiple features of cellular Ca^{2+} handling are preserved at 10 weeks of mechanical overload, indicating this is a model of compensated hypertrophy. The increased Ca^{2+} spark frequency may be due to the augmented SR Ca^{2+} content at 10 weeks. Multiple previous studies show that when the t-tubules are disrupted, Ca^{2+} spark frequency is increased (*e.g.* [8]). This may be due to disrupted interaction of DHPR and RyR, as elements of the DHPR protein stabilize the RyRs at rest [28].

Increased Ca^{2+} spark frequency, an indication of SR Ca^{2+} leak which is observed in heart failure [29], occurs at the extremes of both mechanical overload and mechanical unloading. The enhanced SR Ca^{2+} content could also contribute to the increased Ca^{2+} spark frequency in the 10-week TAC group. Previous studies using the model of severe unloading show that SR Ca^{2+} content is unaffected at 4 weeks [7]. Therefore, it is unlikely that SR Ca^{2+} content changes play a significant role in the changes described in this study. Mechanical unloading, whether moderate or severe, of a normal or failing heart [3], uniformly increased Ca^{2+} spark duration. The mechanisms mediating this remain unclear, but may include altered phosphorylation of RyR clusters [30]. Changes to the width of the Ca^{2+} sparks may be related to altered Ca^{2+} diffusion and buffering properties of these cells. There is evidence that increased Ca^{2+} spark frequency can trigger waves and promote arrhythmias. This is perhaps clearest in the case of atrial fibrillation [31, 32]. The role this plays in the unloaded and overloaded heart should be investigated in future studies.

This study was focused on examining the impact of chronic mechanical load variation on local CICR (the interaction between the Ca^{2+} release trigger and SR Ca^{2+} release), and its structural substrate, the t-tubule network. This system exists in a wider system, of excitation–contraction coupling elements including the Na^{+}/Ca^{2+} exchanger, the action potential, SERCA2a [33]. The assessment of these elements is an important point for future studies. Subcellular Ca^{2+} release may also occur without triggering contraction [34], indicating functions beyond classical excitation–contraction coupling.

T-tubule system as a load-sensitive regulator of CICR

Mechanical unloading appears to induce subtle disorder of the t-tubules, without reduction in their density. These changes are dependent on both the degree and duration of unloading (Fig. 5). Mechanical overload appears to impair their density and regularity. Generally, disordered rather than missing t-tubules appear to be the major form of t-tubule remodelling in small species, whereas in larger species reductions in density appear to play a larger role [35]. The mechanisms mediating the load sensitivity of the t-tubule system are not clear, but a number of candidates are emerging, including BIN-1, T-Cap, JP-2 and others (for a detailed review, see [3]).

Conclusions

Mechanical overloading and unloading result in graded changes to the t-tubule network and local CICR. This supports the notion that the t-tubule system is load sensitive in both degree and duration and that there is a range of physiological loading conditions (degrees and durations) which allow normal t-tubule structure. Importantly, this study supports the notion of a physiological reduction in load which can be associated with normal cardiomyocyte structure and function. While the general hypothesis that mechanical load influences cardiomyocyte t-tubule structure and function has been addressed partially by previous studies, this is the first report to document the specific effect of graded load variation in both positive and negative directions. It is also the first study to examine the effect of moderate mechanical unloading on cellular Ca^{2+} handling.

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Author contributions

Experiments were performed in Harefield Heart Science Centre, Imperial College London, UK. Conception and design of experiments: M.I., M.Y., J.G., C.T. Collection, analysis and interpretation of data: M.I., P. K., U.S., M.N., J.C., S.T., C.V.D., V.T.T., M.Y., J.G., C.T., J.E.C. Drafting the article or revising critically for important intellectual content: M.I., C.V.D., V.T.T., M.Y., J.G., C.T., J.E.C., M.N. All authors have read and approved the final version of this manuscript.

Conflict of interest

No conflict of interest.

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