Intracellular Dyssynchrony of Diastolic Cytosolic [Ca^{2+}] Decay in Ventricular Cardiomyocytes in Cardiac Remodeling and Human Heart Failure

Felix Hohendanner, Senka Ljubojević,* Niall MacQuaide,* Michael Sacherer, Simon Sedej, Liesbeth Biesmans, Paulina Wakula, Dieter Platzer, Sophie Sokolow, André Herchuelz, Gudrun Antoons, Karin Sipido,* Burkert Pieske,* Frank R. Heinzel

**Rationale:** Synchronized release of Ca^{2+} into the cytosol during each cardiac cycle determines cardiomyocyte contraction.

**Objective:** We investigated synchrony of cytosolic [Ca^{2+}] decay during diastole and the impact of cardiac remodeling.

**Methods and Results:** Local cytosolic [Ca^{2+}] transients (1-μm intervals) were recorded in murine, porcine, and human ventricular single cardiomyocytes. We identified intracellular regions of slow (slowCaR) and fast (fastCaR) [Ca^{2+}] decay based on the local time constants of decay (TAU<sub>local</sub>). The SD of TAU<sub>local</sub> as a measure of dyssynchrony was not related to the amplitude or the timing of local Ca^{2+} release. Stimulation of sarcoplasmic reticulum Ca^{2+} ATPase with forskolin or istaroxime accelerated and its inhibition with cyclopiazonic acid slowed TAU<sub>local</sub> significantly more in slowCaR, thus altering the relationship between SD of TAU<sub>local</sub> and global [Ca^{2+}] decay (TAU<sub>global</sub>). Na~/Ca^{2+} exchanger inhibitor SEA0400 prolonged TAU<sub>local</sub> similarly in slowCaR and fastCaR.

FastCaR were associated with increased mitochondrial density and were more sensitive to the mitochondrial Ca^{2+} uniporter blocker Ru360. Variation in TAU<sub>local</sub> was higher in pig and human cardiomyocytes and higher with increased stimulation frequency (2 Hz). TAU<sub>local</sub> correlated with local sarcomere relengthening. In mice with myocardial hypertrophy after transverse aortic constriction, in pigs with chronic myocardial ischemia, and in end-stage human heart failure, variation in TAU<sub>local</sub> was increased and related to cardiomyocyte hypertrophy and increased mitochondrial density.

**Conclusions:** In cardiomyocytes, cytosolic [Ca^{2+}] decay is regulated locally and related to local sarcomere relengthening. Dyssynchronous intracellular [Ca^{2+}] decay in cardiac remodeling and end-stage heart failure suggests a novel mechanism of cellular contractile dysfunction. (*Circ Res. 2013;113:527-538.)*

**Key Words:** calcium ■ diastole ■ heart failure ■ mitochondria ■ myocytes, cardiac ■ ventricular remodeling

Dyssynchrony in heart failure has been used to describe nonsynchronous, abnormal electric activation, and an abnormal sequence of contraction.1 Dyssynchronous activation of ventricular tissue, as it occurs in bundle–branch block, leads to less efficient contraction and worsening of heart failure, whereas resynchronization therapy improves cardiac contractility, morbidity, and mortality in these patients.2 Dyssynchronous contraction is also observed in hearts with diastolic or systolic heart failure in the absence of overt electric conduction defects,3 suggesting dysfunction at the level of the cardiomyocyte.

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Within the cardiomyocyte, Ca^{2+} is the main mediator of contraction and relaxation after electric activation (excitation–contraction coupling). Contraction is initiated by Ca^{2+} release from the sarcoplasmic reticulum (SR), triggered by Ca^{2+} influx through sarcolemmal voltage-dependent Ca^{2+} channels (Ca^{2+}-induced Ca^{2+} release). Simultaneous Ca^{2+}-dependent activation of the myofilaments induces cell shortening. Ca^{2+} is then removed from the cytosol, mainly by the ATP-dependent SR Ca^{2+} pump (SERCA) and the sarcolemmal Na~/Ca^{2+} exchanger (NCX), to allow cell relaxation.

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From the Division of Cardiology, Department of Internal Medicine, Medical University of Graz, Graz, Austria (F.H., S.L., M.S., S.S., P.W., G.A., B.P., F.R.H.); Institute for Biophysics, Medical University of Graz, Graz, Austria (D.P.); Laboratory of Pharmacology, Brussels University School of Medicine, Brussels, Belgium (S.S., A.H.), Laboratory for Experimental Cardiology, University of Leuven, Leuven, Belgium (N.M., L.B., K.S.); and Ludwig Boltzmann Institute for Translational Heart Failure Research, Graz, Austria (S.L., S.S., P.W., B.P., F.R.H.).

Current address for S.S.: UCLA Brain Research Institute, UCLA School of Nursing, Box 956919, 5–946 Factor Bldg, Los Angeles, CA.

*These authors contributed equally to this article.

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Correspondence to Frank R. Heinzel, Division of Cardiology, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. E-mail frank.heinzel@medunigraz.at

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Ca\textsuperscript{2+} controls a variety of other intracellular processes, such as metabolism, gene regulation, and cell death.\textsuperscript{4} As a basis for differential signaling, Ca\textsuperscript{2+} release during Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is tightly controlled in subcellular compartments at junctions of the sarclemmal and sarcoplasmic membrane (coup voyage or Ca\textsuperscript{2+} release units). Diffusion of Ca\textsuperscript{2+} within the cytosol is slow, resulting in intracellular Ca\textsuperscript{2+} gradients during SR Ca\textsuperscript{2+} release.\textsuperscript{5,6} We and others have recently shown that inhomogenous distribution of Ca\textsuperscript{2+} release unit leads to dysynchronous Ca\textsuperscript{2+} release,\textsuperscript{7} contributing to cardiomyocyte dysfunction in myocardial remodeling and heart failure.\textsuperscript{8,9}

After myofilament activation, the rate of Ca\textsuperscript{2+} removal from the cytosol determines the rate of Ca\textsuperscript{2+} diffusion from the myofilaments and cardiomyocyte relaxations. The activity of the Ca\textsuperscript{2+} transporters SERCA and NCX is regulated in functional and structural microdomains, involving kinases, phosphatases, and regulatory proteins.\textsuperscript{10–12} Local regulation of cytosolic Ca\textsuperscript{2+} removal may result in cytosolic diastolic Ca\textsuperscript{2+} gradients attributable to intracellular variation of the rate of [Ca\textsuperscript{2+}] decay.

In the present study, we investigate for the first time intracellular regional differences in the kinetics of cytosolic [Ca\textsuperscript{2+}] decay in beating cardiomyocytes of mice, pigs, and in humans. We explore mechanisms of dysynchronous [Ca\textsuperscript{2+}] decay, its effects on local cardiomyocyte sarcomere relaxation and show increased intracellular variation with cardiac remodeling and heart failure.

**Methods**

An expanded Material and Methods section can be found in the Online Data Supplement.

**Animal Models and Cell Isolation**

All animals were housed and treated according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health). Single cardiomyocytes were isolated as previously described\textsuperscript{7,13} from the left ventricle (LV) of wild-type mice (FVB/N, age 11–20 weeks) or NCX1 heterozygous mice (NCX1\textsuperscript{+/–}) developed by the group of Prof. Herchuelz.\textsuperscript{14} Human LV cardiomyocytes were isolated from end-stage failing hearts removed during heart transplantation or from donor hearts not suitable for transplantation as approved by the Local Ethics Committee (Ref No. 20–277 ex 08/09). None of the donors had a clinical history of heart failure. Donor hearts were classified as nonfailing (preserved ejection fraction in the absence of clinical heart failure symptoms) according to echocardiographic assessment before transplant.

Minimally invasive transverse aortic constriction (TAC) was performed in mice to induce pressure overload and LV hypertrophy as described previously.\textsuperscript{15} Confocal line scan images of cytosolic [Ca\textsuperscript{2+}] transients in cardiomyocytes from a porcine model of chronic myocardial ischemia reported earlier\textsuperscript{4} were analyzed for subcellular dys synchrony in cytosolic [Ca\textsuperscript{2+}] decay.

**Subcellular [Ca\textsuperscript{2+}] Measurements**

Cardiomyocytes were loaded with Fluo3-AM (20 \mu mol/L for 20 minutes), Fluor4-AM (20 \mu mol/L for 35 minutes), or the ratiometric Ca\textsuperscript{2+} indicators Asante-Calcium Red\textsuperscript{16} (10 \mu mol/L for 90 minutes) to quantify cytosolic [Ca\textsuperscript{2+}]. Cardiomyocytes were then kept in Tyrode solution, containing (in mmol/L) NaCl 136, KCl 5, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 1, HEPES 10, and glucose 10; pH was adjusted to 7.35 with NaOH. Cells were transferred to laminin-coated culture dishes on the stage of a confocal microscope (Zeiss, LSM 510 Meta, Jena, Germany), and superfused with Tyrode solution (37°C), containing blebbistatin (10 \mu mol/L, Tocris Bioscience, Bristol, United Kingdom) or butanedione monoxime (10 mmol/L) to avoid cell movement. Line scan images were obtained by repetitive scanning. A scan line was selected parallel to the longitudinal axis, extending across the full length of the cardiomyocyte in the focal plane. All fluorophores were obtained from Life technologies/Invitrogen. All chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated.

**Localization of Mitochondria**

Mitochondria were labeled in Fluor-4 AM loaded cells with Mitotracker Red AM or Tetramethylrhodamine methyl ester (TMRM). Mitotracker Red signal or TMRM signal and Ca\textsuperscript{2+}-dependent fluorescence along the scan line were measured simultaneously in a subset of cells. In TMRM-stained cardiomyocytes, mitochondrial density was quantified by automated thresholding.

**Visualization of Sarcomere and Global Cell Shortening**

In a subgroup of murine cardiomyocytes (SHAM and TAC), cellular contraction was measured in transmitted laser light line scan images in the absence of laminin, blebbistatin, or butanedione monoxime. A subgroup of these cells was loaded with Fluor-4 to simultaneously record confocal Ca\textsuperscript{2+} transients. In another group of mice, the sarcomeres, including the T-tubules, were stained with Alexa Fluor 594 wheat germ agglutinine (10 \mu g/mL for 40 minutes), and the cells were loaded with Fluor-4 AM. Similar recordings were available for analysis from the pig model.\textsuperscript{1} In line scan images, the T-tubule signal appeared as a regular spaced pattern marking the Z-lines of the sarcomeres.\textsuperscript{7}

**Experimental Protocol**

Cardiomyocytes were stimulated in an electric field at the given frequencies until steady state of the cytosolic [Ca\textsuperscript{2+}] transient amplitude. Line scan images of 5 to 10 successive [Ca\textsuperscript{2+}] transients were recorded (baseline). Forskolin (adenylate cyclase agonist; 10 \mu mol/L), cyclopiazonic acid (SERCA-inhibitor; 5 \mu mol/L), SEAD400 (NCX-inhibitor; 0.3 \mu mol/L, provided by Endothem GmbH, Saarbrucken, Germany), 1,2-iodostatin (Na/K-ATPase inhibitor and direct SERCA stimulator\textsuperscript{17}; 1 \mu mol/L for 10 minutes; a gift from Sigma-Tau Industrie Farmaceutiche Riunite S.p.A, Italy), or Ru360 (mitochondrial Ca\textsuperscript{2+} uniporter inhibitor; 5 \mu mol/L, preincubation for 8 minutes; EMD Millipore) were washed-in, and another line scan image of 5 consecutive [Ca\textsuperscript{2+}] transients was recorded at steady state. A subgroup of human cardiomyocytes was stimulated at different frequencies (0.5–2 Hz). In another group of human nonfailing cardiomyocytes, we compared the [Ca\textsuperscript{2+}] transient at 0.5 Hz steady state and the first electrically evoked [Ca\textsuperscript{2+}] transient after a stimulation pause of 10 seconds (postrest). In a subgroup of cardiomyocytes from Sham and TAC mice, the time constant of [Ca\textsuperscript{2+}] decay ([TAU\textsubscript{dec}]) of a caffeine-induced [Ca\textsuperscript{2+}] transient was taken as an estimate of NCX forward mode activity.

**Image Analysis**

[Ca\textsuperscript{2+}]-dependent fluorescence intensity (F) along the scan line was averaged to obtain the global [Ca\textsuperscript{2+}] transient ([E]). Local [Ca\textsuperscript{2+}] transients were calculated for a 1-\mu m sample width around each pixel at the scan line as detailed in the Online Data Supplement. A monoeponential curve was fitted to the decay of the normalized [Ca\textsuperscript{2+}] transient (from 90% amplitude to end of cycle) to derive the time constant of decay for local ([TAU\textsubscript{local}]) and global ([TAU\textsubscript{global}]) [Ca\textsuperscript{2+}] transients. Local [Ca\textsuperscript{2+}] transients were classified as fast [Ca\textsuperscript{2+}] decay (fastCaR; TAU\textsubscript{local}>TAU\textsubscript{global}) or slowCaR ([TAU\textsubscript{local}≤TAU\textsubscript{global}]). The SD of [TAU\textsubscript{local}] (SD\textsubscript{TAU}) along the.
scanned line was taken as a measure of regional variability of the cytosolic \([\text{Ca}^{2+}]\) decay kinetics. Cell length was quantified from the distance between the upper and lower cell margin in the scanned line. In confocal line scans, this distance represents the cell length in the confocal plane (projected cell length). In transmitted light line scans (Online Figure V), the outer margins delineate the overall cell length.

The distance between adjacent sarcomeres (local sarcomere length) was measured in Alexa594-wheat germ agglutinine line scan images as detailed in the Online Data Supplement and Figure 6D. TMRM image stacks were quantified as described in Heinzel8 and the Online Data Supplement.

Statistical Analysis
Data are shown as mean±SEM. For pairwise comparisons (Figures 3, 5, and 8; Online Figure I), a 2-tailed Student \(t\) test was used. Tau of slowCaR and fastCaR were averaged per cell. Comparisons of \(\tau_{\text{global}}\) and \(\tau_{\text{local}}\) in multiple groups (Figures 3D, 6, and 8; Online Figures III and VI) were performed by a 1-way ANOVA followed by a Bonferroni post hoc test. \(\tau_{\text{global}}\) was plotted as a function of \(\tau_{\text{global}}\) for individual cells of each group, and the relationships between \(\tau_{\text{global}}\) and \(\tau_{\text{local}}\) (Figures 3E, 4D, 5B, 7A, and 7B; Online Figures II–V) were compared between groups by multiple linear regression analysis. \(P<0.05\) was considered significant.

**Results**

**Rate of Cytosolic \([\text{Ca}^{2+}]\) Decay Varies in Adjacent Regions**

In murine ventricular cardiomyocytes stimulated at 1 Hz, the time constant of global \([\text{Ca}^{2+}]\) (\(\tau_{\text{global}}\)) was 197±18 ms (n=17 mice; n=48 cells). The time constant of local \([\text{Ca}^{2+}]\) decay (\(\tau_{\text{local}}\)) in the cytosol varied between different regions of the cell (Figure 1A and 1B), with an average range of 237±9 ms between fastest and slowest \(\tau_{\text{local}}\) and an SD (SD\(_{\text{local}}\)) of 17±2 ms (range, 3–55 ms). On average, in regions of fast \([\text{Ca}^{2+}]\) decay (fastCaR; ie, \(\tau_{\text{local}}\geq \tau_{\text{global}}\)), \(\tau_{\text{local}}\) was 19±2% faster as compared with regions with slow \([\text{Ca}^{2+}]\) decay (slowCaR; \(\tau_{\text{local}}\leq \tau_{\text{global}}\)), with a mean \(\tau_{\text{local}}\) of 180±16 versus 216±21 ms, respectively. Analysis of sequential beats revealed little beat-to-beat variability in the local kinetics of \([\text{Ca}^{2+}]\) decay during steady state of the \([\text{Ca}^{2+}]\) transient (Figure 1C). The average widths of contiguous regions with fast and slow \([\text{Ca}^{2+}]\) decay were comparable (4.6±0.2 versus 5.1±0.5 \(\mu\)m, respectively; Figure 1D).

**Variation in the Rate of Cytosolic \([\text{Ca}^{2+}]\) Decay Is Not Related to \([\text{Ca}^{2+}]\) Release or End-Diastolic \([\text{Ca}^{2+}]\)**

We tested whether the kinetics of diastolic cytosolic \([\text{Ca}^{2+}]\) decay was related to the amplitude of the local cytosolic \([\text{Ca}^{2+}]\) transient. Figure 2A shows no significant correlation between \(\tau_{\text{local}}\) and the local \([\text{Ca}^{2+}]\) transient amplitude, although in regions with a \(\tau_{\text{local}}\) close to the mean \(\tau_{\text{global}}\) \([\text{Ca}^{2+}]\) removal seemed slightly faster with higher local \(f_{\text{peak}}\). The low coefficient of correlation (\(R=0.39\)), however, indicates that this only explains a minor part of the observed variation in \(\tau_{\text{local}}\). Diastolic cytosolic \([\text{Ca}^{2+}]\) is a potential determinant

![Figure 1. Variability in the rate of local cytosolic \([\text{Ca}^{2+}]\) decay in murine ventricular myocytes. A. Line scan image of \([\text{Ca}^{2+}]\) transients at 1 Hz. Superimposed at a different scale, the distribution of the time constants of local \([\text{Ca}^{2+}]\) decay (\(\tau_{\text{local}}\), red line) relative to mean \(\tau_{\text{global}}\) along the line (\(\tau_{\text{global}}\), orange). B. Local \([\text{Ca}^{2+}]\) transients at regions with fast (fastCaR, 1) and slow (slowCaR, 2) \([\text{Ca}^{2+}]\) decay (left). Mean \(\tau_{\text{local}}\) for slowCaR and fastCaR in mouse (middle). C. \(\tau_{\text{local}}\) distributions of the 3 example \([\text{Ca}^{2+}]\) transients superimposed reveal little beat-to-beat variability. D. Mean widths of fastCaR and slowCaR are comparable (n=5 cells).](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.117.309338)
of the cytosolic [Ca^{2+}] buffering reserve. Therefore, we investigated the impact of local differences in end-diastolic [Ca^{2+}] on TAU_local of the local [Ca^{2+}] transient decay independent of regional variations in the reporting Ca^{2+} dye by using Asante Ca^{2+} Red as a ratiometric Ca^{2+} indicator (Figure 2B–2D; n=9 cardiomyocytes). As shown in Figure 2D, TAU_local was not related to the mean local end-diastolic [Ca^{2+}] averaged during the last 30 ms before the stimulus.

Modulation of SERCA Activity Has Significantly Stronger Effects in Regions of Slow [Ca^{2+}] Decay

Forskolin stimulates SERCA activity via an adenylate cyclase/protein kinase A–dependent pathway. With forskolin, the maximal amplitude of the [Ca^{2+}] transient was significantly increased (6.5±0.7 versus 4.9±0.5 F/F_0; Figure 3A and 3B; n=17). Forskolin accelerated the cytosolic [Ca^{2+}] decay (TAU_global) significantly more in slowCaR than in fastCaR, as reflected by an inverse linear relationship between the relative TAU_local at baseline and the decrease in TAU_local with application of forskolin (Figure 3C). Similar effects were observed when SERCA activity was increased by is-taroxime or by increased SR Ca^{2+} release after a stimulation pause (postrest potentiation; Figure 3D; see also Online Data Supplement and Online Figure IV for details). SERCA inhibition by cyclopiazonic acid significantly slowed the whole-cell cytosolic [Ca^{2+}] decay (Figure 3D; TAU_global: 264±29 versus 189±13 ms at baseline). Again, the local cytosolic [Ca^{2+}] decay was significantly more affected in slowCaR than in fastCaR (Figure 3D).

The distribution of TAU_local at baseline was normally distributed (Shapiro–Wilk test), thus it can be described by the spatially averaged TAU_global and SD_TAU. TAU_global was linearly correlated with SD_TAU (Figure 3E, black line; R= 0.65; P<0.05; n=28 cells). If the modulation of SERCA activity affects SD_TAU, regardless of changes in TAU_global, then the relationship between TAU_global and SD_TAU should be altered. Indeed, in the presence of forskolin, the relationship between TAU_global and SD_TAU was significantly less steep (Figure 3E; n=17 cells; P<0.05 versus baseline), indicating less influence of intracellular variation in TAU_local on TAU_global. In contrast, in the presence of cyclopiazonic acid, SD_TAU was significantly higher than at baseline (45±7 versus 26±5 ms; n=11 cells; P<0.01), the slope tended to be steeper than at baseline (P=0.16), and TAU_global was more strongly related to SD_TAU (R=0.90).

Variation in the Rate of Cytosolic [Ca^{2+}] Decay Was Unchanged With Altered NCX Activity

The effect of inhibition of the sarcolemmal NCX with SEA0400 on local [Ca^{2+}] decay kinetics was evaluated in murine cardiomyocytes (Online Figure IIA). With the addition of SEA0400, TAU_global and SD_TAU were significantly increased. TAU_local was similarly prolonged in fastCaR and slowCaR, so that the relationship between TAU_global and SD_TAU was unchanged (Online Figure IIB). We confirmed these findings in the TAC mouse model, which showed increased NCX1 protein expression and forward mode activity (Online Figure III). NCX inhibition with SEA0400 had no effect on the relationship between TAU_global and SD_TAU in TAC. Furthermore, in NCX1−/− mice, the relationship between TAU_global and SD_TAU was unchanged. See Online Data Supplement for a detailed description of NCX1-related experiments.

Rate of Regional Cytosolic [Ca^{2+}] Decay and Mitochondria

We compared the distribution of mitochondria visualized with the fluorescent dye Mitotracker Red with...
the kinetics of local cytosolic [Ca$^{2+}$] decay in the same cell. The association of structures and Ca$^{2+}$ dynamics in 2-dimensional images is complicated by the potential effects of close by but out-of-plane structures.\cite{7,19} However, despite this limitation, regions with increased density of the mitochondrial signal were significantly more often associated with fast cytosolic [Ca$^{2+}$] decay (Figure 4A). We used Ru360 to block mitochondrial Ca$^{2+}$ uptake via the mitochondrial Ca$^{2+}$ uniporter. In the majority of cardiomyocytes (n=8/14), Ru360 induced a prolongation of TAU$_{global}$ (242±32 versus 187±31 ms; P<0.01). In these cells, the prolongation of cytosolic [Ca$^{2+}$] decay was significantly higher in fastCaR than in slowCaR (Figure 4B and 4C). The relationship between TAU$_{global}$ and SD$_{TAU}$ tended to be steeper with Ru360 (Figure 4D; P=0.083 for difference in regression lines).

**Intracellular Variation in the Rate of [Ca$^{2+}$] Decay in Porcine and Human Myocardium**

We compared the intracellular variation of local cytosolic [Ca$^{2+}$] decay kinetics in mouse, pig, and human ventricular cardiomyocytes (Figure 5A). TAU$_{global}$ was significantly different among all species (human: 510±77 ms, n=4; pig: 251±11 ms, n=54; mouse: 137±11 ms, n=33; all P<0.001). Average SD$_{TAU}$ was larger in human (169±19 ms) versus pig (62±5 ms) versus mouse (12±7 ms), and SD$_{TAU}$ was linearly correlated with TAU$_{global}$ within the groups (Figure 5B). The regression curves of SD$_{TAU}$ and TAU$_{global}$ however, were significantly different among species.

We and others have previously shown that in porcine,\cite{7} as well as in human,\cite{20} cardiomyocytes Ca$^{2+}$ release after depolarization is delayed in some regions of the cell, related to a lower density of T-tubules with couplons in these regions. In porcine ventricular myocytes, the timing of SR Ca$^{2+}$ release (time to half maximal release) had no impact on TAU$_{local}$ (Figure 5C, left). Our results suggest that the distribution of T-tubules does not influence the observed variation in the rate of cytosolic [Ca$^{2+}$] decay. However, delayed local Ca$^{2+}$ release translates into delayed initiation of local Ca$^{2+}$ removal. Figure 5C (right) demonstrates that dysynchronous Ca$^{2+}$ release adds to the regional differences in the time to near complete cytosolic [Ca$^{2+}$] decay (RT$_{90}$). We conclude that dysynchrony in Ca$^{2+}$ release adds to the dysynchrony in the timing to restore cytosolic resting [Ca$^{2+}$] (Figure 5C, right), but not to the variation in the rate of local [Ca$^{2+}$] removal (Figure 5C, left).

![Figure 3. Sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) modulation predominantly affects regions of slow [Ca$^{2+}$] decay. A, Line scan image of murine cardiomyocyte at baseline (top) and in presence of forskolin (bottom). Red line indicates distribution of TAU$_{local}$; below the linescans, example [Ca$^{2+}$] transients. B, Examples of local [Ca$^{2+}$] transients showing a stronger effect of forskolin in regions of slow (slowCaR) vs fast (fastCaR) [Ca$^{2+}$] decay at baseline. C, Inverse linear relationship between change in TAU$_{local}$ with forskolin and TAU$_{local}$ at baseline (symbol=pixel on scan line; 2 example cells, significant inverse linear correlation (red) in 13 out of 16 cells). D, Modulation of SERCA activity by means of forskolin, istaroxime, after rest potentiation (human cardiomyocytes) and cyclopiazonic acid (CPA) preferentially affects slowCaR (see text for details). E, Linear correlation between TAU$_{global}$ and SD$_{TAU}$ at baseline is altered by forskolin and CPA, and reflects significantly less (forskolin) or more (CPA) variability in local [Ca$^{2+}$] decay kinetics as measured by SD$_{TAU}$ (each symbol=1 cell; see text for details).](http://circres.ahajournals.org/)<br/>
Cardiomyocytes typically show frequency-dependent acceleration of relaxation, attributed to CaMKII-dependent SERCA activation. As expected, TAUglobal was significantly faster with increased stimulation frequency in human cardiomyocytes (Figure 5B, right). However, this is associated with increased variability in local [Ca2+] decay as reflected by SD TAU. With higher stimulation frequency, the relationship between TAUglobal and SD TAU was significantly less steep, indicating less influence of SD TAU on TAUglobal.

Subcellular Mechanical Dyssynchrony

We measured whole-cell contraction in transmitted light line scan images (Figure 6A) and found a significant linear correlation between TAU global of the [Ca2+] transient measured in the confocal plane and the time constant of whole-cell relengthening (Figure 6B). Murine cardiomyocytes have a very regular T-tubule network with the transverse tubules following the Z-lines of the sarcomeres at a regular spacing of ≈2 μm in the line scan images. We used Alexa594-wheat germ agglutinin to stain the transverse tubules and thereby visualize local sarcomere shortening during electric stimulation (Figure 6C). The time constant of local sarcomere relengthening calculated from the sarcomere length at 5 time points during relaxation (Figure 6D) correlated significantly with TAU local of cytosolic [Ca2+], indicating that dyssynchrony of the decay of cytosolic [Ca2+] contributes to dyssynchronous intracellular sarcomere relengthening. A significant linear correlation between TAU local of sarcomere relengthening and TAU local of [Ca2+] (Figure 6E) was found in 5 out of 6 cells (R=0.65±0.06).

Dyssynchrony in Cytosolic [Ca2+] Decay Is Increased in Myocardium With Severe Contractile Dysfunction

We investigated the intracellular variation of cytosolic [Ca2+] decay in ventricular cardiomyocytes in a murine model of heart failure after 6 weeks of TAC. As shown in Figure 7A, SDtau was significantly higher in TAC (5 hearts, n=9 cells) as compared with sham-operated animals (5 hearts, n=8 cells). The relationship between TAU global and SDlocal of the [Ca2+] transient (Online Figure VD) was significantly different between TAC and Sham (Figure 7A, middle). In TAC, cardiomyocyte relengthening was significantly slower than in Sham (Online Figure VA–VC). Similarly, in TAC, TAU global of cell relengthening tended to increase as a function of TAU global and SDlocal of the [Ca2+] transient (Online Figure VD). Projected cell length in the confocal plane of the line scan was significantly higher in TAC versus Sham (137±22 versus 72±10 μm), reflecting cardiomyocyte...
hypertrophy reported earlier in this model. SD TAU was significantly correlated to projected cell length (Figure 7C; R=0.54; P<0.05).

Similarly, in hypertrophied cardiomyocytes from the infarct border zone of a porcine model of chronic myocardial ischemia, SD TAU was significantly larger, whereas TAU global was comparable with Sham. Similar to our findings in the mouse, the addition of forskolin accelerated cytosolic $[Ca^{2+}]$ decay significantly more in slowCaR compared with fast-CaR (see Online Data Supplement for details). Finally, we examined subcellular $[Ca^{2+}]$ decay in LV cardiomyocytes isolated from end-stage failing human hearts (3 hearts, n=7 cells), donor hearts from patients without clinical history of heart failure but with echocardiographic signs of cardiac remodeling and mild-to-moderately reduced ejection fraction (n=2 hearts, n=4 cells) and donor hearts (n=4, n=18) without echocardiographic signs of cardiac remodeling or dysfunction (see Online Table I for details). Figure 7B shows that SD TAU and TAU global were unchanged in early cardiac remodeling but both significantly increased in end-stage failing hearts. SD TAU tended to increase with projected cell length (Figure 7C, right).

**Figure 5. Dyssynchrony of cytosolic $[Ca^{2+}]$ decay in pigs and humans.** A, Example $[Ca^{2+}]$ transients from mouse, pig, and human nonfailing ventricular cardiomyocytes with TAU local distribution (red line). Dyssynchrony (SD TAU) is higher in pigs and the highest in human cardiomyocytes (see text for details). B, Left, Relationship between TAU global and SD TAU is significantly different between species. Right, In human nonfailing cardiomyocytes, intracellular variation in the rate of local $[Ca^{2+}]$ decay increases with higher frequency of stimulation, whereas TAU global decreases. C, Time constant of cytosolic $Ca^{2+}$ removal (TAU local, left) and timing of 90% $Ca^{2+}$ removal (RT90) as a function of the onset of $Ca^{2+}$ release (TF50) in pig cardiomyocytes. Delayed local $Ca^{2+}$ release does not influence the rate of $[Ca^{2+}]$ decay but prolongs RT90 (right) and, thus, adds to the dyssynchrony in restoring resting cytosolic $[Ca^{2+}]$. Error bars=SD.

**Dyssynchrony Related to Altered Mitochondrial Density**

We compared mitochondrial signal density in Sham and TAC mice as well as in human nonfailing cardiomyocytes. Mitochondrial signal density was higher in human as compared with murine nonfailing cardiomyocytes (Online Figure VIIIB). Mitochondrial signal density was also increased in 6-week TAC versus Sham mice (Online Figure VIIIC). Increased SD TAU was significantly related to mitochondrial signal density recorded in the same cells (Online Figures VIIIB and VIIIC).

**Discussion**

In cardiac myocytes, coordinated $Ca^{2+}$ release from the SR determines cellular contraction. $Ca^{2+}$-induced $Ca^{2+}$ release is regulated locally in subcellular microdomains. Recent evidence has shown that disruption of this intracellular organization, for example, by the loss of T-tubules, contributes to contractile dysfunction in chronic ischemia, myocardial hypertrophy, and heart failure.

We provide the first quantitative evidence for regional differences in the subcellular regulation of $[Ca^{2+}]$ decay in the cytosol of cardiac myocytes during diastole. The rate of $[Ca^{2+}]$
Intracellular variation in the rate of cytosolic $[\text{Ca}^{2+}]$ decay was significantly increased in animal models of myocardial hypertrophy (after TAC) and chronic myocardial ischemia. Dyssynchrony was also significantly increased in end-stage human heart failure, associated with slowed global $[\text{Ca}^{2+}]$ decay.

In the present study, we used the SD (SD$_{TAU}$) as an indicator of spatial variability in the rate of cytosolic $[\text{Ca}^{2+}]$ decay. It seems reasonable to interpret SD$_{TAU}$ in the context of mean TAU (TAU$_{global}$). Indeed, we found a positive linear relationship between these 2 parameters in cardiomyocytes under similar conditions. However, interventions and conditions leading to altered intracellular $\text{Ca}^{2+}$ trafficking had differential effects on SD$_{TAU}$ and TAU$_{global}$, resulting in significant alterations in their relationship and confirming SD$_{TAU}$ and TAU$_{global}$ as 2 complementary parameters in the regulation of cytosolic $[\text{Ca}^{2+}]$ decay.

A variety of processes are regulated by local cytosolic $[\text{Ca}^{2+}]$, and different rates of cytosolic $[\text{Ca}^{2+}]$ decay may result in dyssynchrony of $\text{Ca}^{2+}$-mediated processes during the cardiac cycle, as we have documented with local sarcomere relengthening. In addition to the rate of local $[\text{Ca}^{2+}]$ decay, dyssynchrony may be influenced by variation in the onset of local $\text{Ca}^{2+}$ removal, which is determined by the onset of $\text{Ca}^{2+}$ release into the cytosol. We demonstrate in Figure 5C that the onset of local $\text{Ca}^{2+}$ release significantly influences the timing of restoration of cytosolic $[\text{Ca}^{2+}]$ (RT$_{90}$), but not the rate of $[\text{Ca}^{2+}]$ decay. Therefore, the onset of $\text{Ca}^{2+}$ release (and thus removal) and the rate of cytosolic $[\text{Ca}^{2+}]$ decay are independent determinants of dyssynchrony (Figure 5C).

SR $\text{Ca}^{2+}$ reuptake via SERCA and NCX1-mediated $\text{Ca}^{2+}$ extrusion are the main pathways of $\text{Ca}^{2+}$ removal from the cytosol in cardiac myocytes. As discussed in more detail in the Online Data Supplement, NCX1 activity did not contribute to dyssynchrony in murine nonfailing and TAC cardiomyocytes. However, because NCX1 activity in human myocardium has been reported to contribute to an even larger extent to cytosolic $\text{Ca}^{2+}$ removal, the role of NCX for dyssynchrony in human myocardium remains to be investigated.

Dyssynchronous $\text{Ca}^{2+}$ removal could potentially be explained by an inhomogenous distribution of SERCA2a protein along the SR. Our experiments in intact primary cardiomyocytes did not allow for simultaneous visualization of SERCA distribution. However, previous reports...
indicate a homogenous sarcomeric distribution pattern of SERCA2a. In cardiomyocytes, SERCA activity is highly regulated (see Online Data Supplement for a detailed discussion). In the present study, local cytosolic [Ca\(^{2+}\)] decay in slowCaR was significantly more sensitive to SERCA stimulation and inhibition (Figure 3; Online Figure IV), indicating a larger role of SERCA in removing cytosolic Ca\(^{2+}\) in slow-CaR regions versus fastCaR regions. The rate of SR Ca\(^{2+}\) reuptake by SERCA depends on cytosolic [Ca\(^{2+}\)], which could imply that local Ca\(^{2+}\) release may affect the kinetics of local SR Ca\(^{2+}\) reuptake. However, neither the timing of local Ca\(^{2+}\) release (Figure 5C) nor the local peak amplitude of cytosolic [Ca\(^{2+}\)] (Figure 2A) contributed significantly to the observed variance in the local kinetics of cytosolic [Ca\(^{2+}\)] decay.

In the present study, we observed only little beat-to-beat variability in the distribution of slowCaR and fastCaR during steady-state stimulation. In addition, faster Ca\(^{2+}\) turnover with higher stimulation frequency (Figure 5B) increased the spatial variability in TAU\(_{\text{local}}\). These observations may indicate that the variability in TAU\(_{\text{local}}\) is related to the distribution of intracellular structures. We found that regions of fast [Ca\(^{2+}\)] decay were more often associated with regions of increased mitochondrial signal density (Figure 4). The role of mitochondrial Ca\(^{2+}\) uptake in regulating excitation–contraction coupling is not completely understood (see Online Data Supplement for a more detailed discussion). Mitochondria and the endoplasmatic reticulum form narrow compartments, which are constituted by direct links between the endoplasmatic reticulum membrane and the outer mitochondrial membrane (tethers). SERCA is sensitive to mitochondrial ATP production and the organization of mitochondria and SR may directly influence the rate of SR Ca\(^{2+}\) reuptake. However, the role of metabolic regulation for subcellular differences in cytosolic [Ca\(^{2+}\)] decay remains to be determined.

Because the time course of cytosolic [Ca\(^{2+}\)] decay correlates with cardiomyocyte relaxation (Figure 6A and 6B), we hypothesized that local variation in the decay of cytosolic [Ca\(^{2+}\)] may be associated with different rates of sarcomere relengthening within the same cardiomyocyte, as confirmed in Figure 6E. Interestingly, several authors have shown before that sarcomere relaxation is not uniform in striated muscle (see Reference 31 for review). Edman and Flitney hypothesized that nonuniform changes in sarcomere lengths are mainly because of regional differences in the rate of Ca\(^{2+}\) removal. In the present study, we provide the evidence for regional inhomogeneities in [Ca\(^{2+}\)] decay giving rise to dysynchronous sarcomere relengthening.
Dyssynchronous sarcomere relengthening can influence the kinetics of muscle tension decay. Stern et al suggested that local diastolic Ca\textsuperscript{2+} gradients (related to spontaneous Ca\textsuperscript{2+} waves) may impair relaxation by increasing diastolic tension, as well as reducing contraction during the subsequent beat. Slow decay of [Ca\textsuperscript{2+}] from the cytosol may reactivate crossbridge cycling, and it is conceivable that the subcellular synchrony of cytosolic [Ca\textsuperscript{2+}] decay and sarcomere relengthening determine the efficacy of cardiomyocyte relaxation.

In multicellular preparations and at increased mechanical load myofilament, cross-bridge kinetics are the major rate determining step in relaxation. Because our recordings were performed in isolated unloaded cardiac myocytes, the correlation between parameters of intracellular [Ca] decay (ie, TAU\textsubscript{global} or SD\textsubscript{Tau}) and the rate of relaxation (Online Figure VD), thus, may be confounded in vivo by slower myofilament relaxation. Therefore, the role of dyssynchronous Ca\textsuperscript{2+} reuptake for mechanical relaxation in vivo remains to be determined. However, cytosolic Ca\textsuperscript{2+} removal is the initiating step for relaxation, and the rate of cytosolic Ca\textsuperscript{2+} removal does influence relaxation kinetics in isolated cells. Multicellular preparations, and also in vivo. Interestingly, if Ca\textsuperscript{2+} is removed (nearly) instantaneously, mechanical nonuniformity in relengthening of sequential sarcomeres may even facilitate myofilament relaxation. The spatial differences in local Ca\textsuperscript{2+} removal kinetics we describe here likely also result in heterogeneity of Ca\textsuperscript{2+} removal of contiguous sarcomeres of the same myofibril (see Online Data Supplement for details). However, although some extent of dyssynchrony may provide a benefit for relaxation in physiological conditions, we clearly show that increased dyssynchrony is associated with slowed [Ca\textsuperscript{2+}] decay and cardiomyocyte relaxation. Additionally, passive stretching of weaker (yielding) sarcomeres by their contracted neighbors results in reduced myofilament Ca\textsuperscript{2+} binding, and Ca\textsuperscript{2+} surges released from the myofilaments during nonuniform contraction have been identified as a cause of arrhythmias. Thus, an increased dyssynchrony in intracellular [Ca\textsuperscript{2+}] decay and sarcomere relengthening may in several ways impair cardiomyocyte function.

A significantly increased level of dyssynchrony in cytosolic [Ca\textsuperscript{2+}] decay was observed in LV cardiomyocytes from murine hypertrophied failing hearts after TAC (Figure 7A), associated with slowed cardiomyocyte relengthening in TAC (Online Figure VC). Similarly, in the porcine model of chronic regional myocardial ischemia, a significant, albeit less pronounced, increase in dyssynchrony was observed in cardiomyocytes from the infarct border zone (Online Data Supplement). Finally, we could confirm in human hearts that an elevated level of dyssynchrony is characteristic for stage D heart failure.

Both models and human end-stage heart failure are associated with cardiomyocyte hypertrophy as a result of myocardial remodeling (Figure 7C). Although confocally derived cell length in 1 plane is only an estimate of total cell size, our data indicate that dyssynchrony in cytosolic [Ca\textsuperscript{2+}] decay is related to cellular hypertrophy.

In electron micrographs, mitochondrial density is higher in human as compared with rodent hearts. Similarly, we found an increased mitochondrial signal density in nonfailing human as compared with murine cardiomyocytes (Online Figure VIII). In TAC mice, mitochondrial signal density was significantly higher than in Sham and correlated with increased dyssynchrony of Ca\textsuperscript{2+} reuptake. Mitochondrial alterations in cardiac remodeling have been described before, with an increased mitochondrial mass reported in some but also a decrease in mitochondrial size (mitochondrial fission) observed in other models. An increase in mitochondrial volume indicating mitochondrial remodeling was also found in electron micrographs in the present TAC model. Despite this evidence, it is currently unclear why mitochondrial remodeling with increased mitochondrial density is associated with more dyssynchrony, and we cannot exclude that additional mechanisms leading to heterogeneity in local mitochondrial or SR function contribute to dyssynchronous cytosolic Ca\textsuperscript{2+} removal.

Interestingly, dyssynchrony in Ca\textsuperscript{2+} removal was not increased in the hearts from donors with no clinical history of heart failure but adverse LV remodeling and mild-to-moderate reduced ejection fraction, equivalent to heart failure stage B. Because global LV function was also only mildly reduced in the porcine model, our results highlight the close association between regional myocardial function and cellular function of the cardiomyocytes.

In summary, we show that dyssynchronous diastolic [Ca\textsuperscript{2+}] decay within individual cardiomyocytes contributes to dyssynchronous intracellular sarcomere relengthening. Intracellular dyssynchrony of [Ca\textsuperscript{2+}] decay is increased in animal models and in human heart failure and represents a potential new mechanism underlying contractile dysfunction in cardiac remodeling.

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Novelty and Significance

What Is Known?

• Cytosolic [Ca]2+ transients determine the rate of contraction and relaxation of cardiac myocytes.
• On excitation, Ca2+ release from the intracellular stores (sarcoplasmic reticulum) into the cytosol is highly controlled in subcellular microdomains (couplons).
• Removal of Ca2+ from the cytosol initiates myofilament relaxation and is mainly mediated by the sarcoplasmic reticulum Ca2+ pump and the sarcolemmal Na+/Ca2+ exchanger.

What New Information Does This Article Contribute?

• The rate of Ca2+ removal from the cytosol varies between different regions within a cardiac myocyte.
• Intracellular regions with slow Ca2+ removal respond more strongly to changes in sarcoplasmic reticulum Ca2+ pump activity, whereas fast Ca2+ removal sites are associated with mitochondria and are more sensitive to changes in mitochondrial Ca2+ uptake.
• The kinetics of local sarcomere relengthening are related to the local rate of Ca2+ removal.

• In experimental cardiac remodeling and in end-stage human heart failure, increased intracellular variation in the rate of local Ca2+ decay leads to increased dyssynchrony in cytosolic Ca2+ removal, associated with slowed cardiomyocyte relaxation.

Local control of Ca2+ release from the intracellular stores during excitation–contraction coupling has been studied intensively, but the regulation of calcium within microdomains of cardiac myocytes remains unclear. We now report that removal of Ca2+ from the bulk cytosol is compartmentalized, and that the rate of local cytosolic Ca2+ decay directly affects the relaxation of adjacent sarcomeres. In a murine and a porcine model of cardiac remodeling and in end-stage human failing hearts, intracellular Ca2+ removal is more dyssynchronous and is associated with slower cardiomyocyte relaxation. Dyssynchronous intracellular Ca2+ removal represents a novel mechanism underlying contractile dysfunction in cardiac remodeling.
Intracellular Dyssynchrony of Diastolic Cytosolic [Ca\textsuperscript{2+}] Decay in Ventricular Cardiomyocytes in Cardiac Remodeling and Human Heart Failure

Felix Hohendanner, Senka Ljubojevic, Niall MacQuaide, Michael Sacherer, Simon Sedej, Liesbeth Biesmans, Paulina Wakula, Dieter Platzer, Sophie Sokolow, André Herchuelz, Gudrun Antoons, Karin Sipido, Burkert Pieske and Frank R. Heinzel

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Intracellular Dyssynchrony of Diastolic Cytosolic \([\text{Ca}^{2+}]\) Decay in Ventricular Cardiomyocytes in Cardiac Remodeling and Human Heart Failure

**SUPPLEMENTAL MATERIAL**

Felix Hohendanner\(^1\), Senka Ljubojevi\(\acute{c}\)\(^1,5\)*, Niall MacQuaide\(^4\)*, Michael Sacherer\(^1\), Simon Sedej\(^1,5\), Liesbeth Biesmans\(^4\), Paulina Wakula\(^1,5\), Dieter Platzer\(^2\), Sophie Sokolow\(^3\), André Herchuelz\(^3\), Gudrun Antoons\(^3\), Karin Sipido\(^5,\)*, Burkert Pieske\(^5,\)*, Frank R. Heinzel\(^1,5,\#\)

\(^1\)Division of Cardiology, Medical University of Graz, Graz, Austria; \(^2\)Institute for Biophysics, Medical University of Graz, Graz, Austria; \(^3\)Laboratory of Pharmacology, Brussels University School of Medicine, Brussels, Belgium; \(^4\)Laboratory for Experimental Cardiology, University of Leuven, Leuven, Belgium; \(^5\)Ludwig Boltzmann Institute for Translational Heart Failure Research, Graz, Austria;

*equal contribution

\(^\#\)correspondence to Frank R. Heinzel, MD, PhD. Div. of Cardiology, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. Email: frank.heinzel@medunigraz.at; Tel. +43 316 385 80772; Fax. +43 316 385 13733

Extended Methods

**Animal Models and Cell Isolation**

All animals were housed and treated according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health, U.S.A.). Single cardiomyocytes were isolated as previously described\(^1,2\) from the left ventricle (LV) of wild-type (WT) mice (FVB/N, age 11 – 20 weeks) or NCX1 heterozygous mice (NCX1\(^+/−\)) developed by the group of Prof. Herchuelz\(^3\). Human LV cardiomyocytes were isolated from end-stage failing hearts removed during heart transplantation or from donor hearts not suitable for transplantation as approved by the local ethics committee (Ref. No. 20-277 ex 08/09). None of the donors had a clinical history of heart failure. Donor hearts were classified as non-failing (preserved ejection fraction, EF) and in remodeling (reduced EF in the absence of clinical heart failure symptoms) according to echocardiographic assessment before explant.

Minimally invasive transverse aortic constriction (TAC) was performed to induce pressure-overload and LV hypertrophy as described previously\(^4\). Briefly, ketamine/xylazine-anaesthetized, self-ventilating WT mice underwent standardized TAC using a 27-gauge needle. A horizontal incision at the jugulum was used to visualize the aorta. The 27-gauge needle was placed parallel to the aorta...
and tied against the transversal part of aorta (between the right innominate artery and the left common carotid artery). After removal of the needle, the skin was closed. A subgroup of mice underwent a sham procedure consisting of aortic exposure without ligation. All mice received analgesic metamizol-sodium (1 ml dissolved in 100 ml drinking water) for 1 week. For all experimental protocols similar number of males and females were used in each group to avoid potential gender-related bias. All animal procedures were approved by the responsible government agency (BMWF-66.010/0062-II/10b/2010).

Confocal line scan images of cytosolic $[\text{Ca}^{2+}]$ transients in cardiomyocytes from a porcine model of chronic myocardial ischemia reported earlier were analysed for subcellular dyssynchrony in cytosolic $[\text{Ca}^{2+}]$ decay. In the porcine chronic ischemia model, (N=13; weight: 51±2 kg) described earlier, a copper coated stent was placed in the left circumflex artery and cells were isolated (47±1 days after stent implantation) from the peri-infarct region. Healthy pigs were used as control-group (N=15; weight: 55±4 kg).

**Subcellular $[\text{Ca}^{2+}]$ measurements**

Cardiomyocytes were loaded with Fluo3-AM (20 µmol/L for 20 min), Fluo4-AM (20 µmol/L for 35 min), or the ratiometric $\text{Ca}^{2+}$ indicators Asante-Calcium Red (10 µmol/L for 90 min) to quantify cytosolic $[\text{Ca}^{2+}]$. Cardiomyocytes were then washed and kept in Tyrode’s solution, containing (in mmol/L) NaCl 136, KCl 5, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, glucose 10; pH was adjusted to 7.35 with NaOH. Cells were transferred to laminin-coated culture dishes on the stage of a confocal microscope (Zeiss, LSM 510 Meta, Jena, Germany), and superfused with Tyrode’s solution (37°C), containing blebbistatin (10 µmol/L, Tocris Bioscience, Bristol, UK) or butanedione monoxime (BDM, 10 mmol/L) to avoid cell movement. Line scan images were obtained by repetitive scanning (excitation at 488 nm; emission at 525 nm for Fluo-3 and Fluo-4, and 525 nm and 650 nm for Asante-Calcium Red; 1.54 ms/line, pixel size 0.12-0.38 µm). A scan line was selected parallel to the longitudinal axis, extending across the full length of the cardiomyocyte in the focal plane. All fluophores were obtained from Life technologies/Invitrogen, USA. All chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated.
Localization of mitochondria

Mitochondria were labelled in Fluo-4-AM loaded cells with Mitotracker Red AM (MTR, 25 µmol/L for 35 min, 2x washing) or Tetramethylrhodamine, methyl ester (TMRM, 1 µmol/L for 10 min, 1x wash). MTR signal or TMRM signal (both excitation at 543 nm; emission at 644 nm) and Ca²⁺-dependent fluorescence along the scan line were measured simultaneously in a subset of cells. In TMRM stained cardiomyocytes a stack of 5 XY images (pixel size between 0.1 and 0.2 µm) spaced 1 µm in the Z-direction were recorded around the equatorial plane of the cardiomyocyte and the centered plane was used to quantify mitochondrial density.

Visualization of sarcomere and global cell shortening

In a subgroup of murine cardiomyocytes (Sham and TAC), cellular shortening during contraction was measured in transmitted laser light line scan images in the absence of laminin, blebbistatin or BDM. A subgroup of these cells was loaded with Fluo-4 to simultaneously record confocal Ca²⁺ transients. In another group of mice, the sarcolemma including the T-tubules was stained with Alexa Fluor 594 WGA (10 µg/ml for 40 min) and the cells were loaded with Fluo-4 AM. Similar recordings were available for analysis from the pig model. In line scan images, the T-tubule signal appeared as a regular spaced pattern marking the Z lines of the sarcomeres.

Experimental protocol

Cardiomyocytes were stimulated in an electrical field at the given frequencies until steady state of the cytosolic [Ca²⁺] transient amplitude. Line scan images of 5-10 successive [Ca²⁺] transients were recorded (baseline). Forskolin (adenylyl cyclase agonist; 10 µmol/L), cyclopiazonic acid (CPA; SERCA-inhibitor; 5 µmol/L), SEA0400 (NCX-inhibitor; 0.3 µmol/L, kindly provided by Endotherm GmbH, Saarbrucken, Germany), istaroxime (Na⁺/K⁺-ATPase inhibitor and direct SERCA stimulator; 1 µmol/L for 10 min; a kind gift from Sigma-Tau Industrie Farmaceutiche Riunite S.p.A, Italy) or Ru360 (mitochondrial Ca²⁺ uniporter inhibitor; 5 µmol/L, pre-incubation for 8 min; EMD Millipore, USA) were washed-in as required by the protocol and another line scan image of 5 consecutive transients was recorded at steady state. A subgroup of human cardiomyocytes was also stimulated at different frequencies (0.5 - 2 Hz). In another group of human non-failing cardiomyocytes we compared the [Ca²⁺] transient at 0.5 Hz steady state and the first electrically evoked [Ca²⁺] transient after a stimulation pause of 10 seconds (post-rest). In a subgroup of cardiomyocytes from Sham and TAC mice, following the steady state recording ±SEA0400 external solution was rapidly switched to a Na⁺- and Ca²⁺-free solution to inhibit Na⁺- and Ca²⁺ transport via the sarcolemmal NCX (solution contained in mmol/L: LiCl 91, LiOH 21, KCl 4, MgCl₂ 1, HEPES 20, EGTA 10, glucose 10; pH 7.4 with LiOH). After equilibrium of cytosolic [Ca²⁺] (i.e. of transsarcoplasmic Ca²⁺ fluxes), Tyrode’s solution (with Na⁺ and Ca²⁺) was briefly reintroduced (2 sec. by rapid perfusion) followed by wash-in of caffeine (20 mmol/L) for 10 sec. The time constant of [Ca²⁺] decay in the presence of caffeine (TAU_caff) was taken as an estimate of NCX forward mode activity.
Image analysis

[Ca^{2+}]-dependent fluorescence intensity (F) along the scan line was averaged to obtain the global [Ca^{2+}] transient (F_g). The onset of F_g was defined as the start of Ca^{2+} release (t_0). Local [Ca^{2+}] transients were calculated for a 1 µm sample width around each pixel at the scan line (except for Online Fig. VII, see Suppl. Results for details). F for each pixel on the scan line was normalized to the average F of the last 30 ms before t_0 at that pixel position (F_0). F_g was normalized to the average F_0.

For each local [Ca^{2+}] transient the time to half-maximal amplitude (T_{F50} \text{,} t_{peak}) peak amplitude (F_{peak}) and time to 90% decay (R_{T90} \text{,} from t_0) were calculated. A mono-exponential curve was fitted to the decay of the [Ca^{2+}] transient (from 90% amplitude to end of cycle) to derive the time constant of decay for local (T_{AU_{local}}) and global (T_{AU_{global}}) [Ca^{2+}] transients. Local [Ca^{2+}] transients were classified as transients with fast [Ca^{2+}] decay (fastCaR, T_{AU_{local}} \leq T_{AU_{global}}) or slowCaR (T_{AU_{local}} > T_{AU_{global}}). The standard deviation of T_{AU_{local}} along the scanned line was taken as a measure of regional variability of the cytosolic [Ca^{2+}] decay kinetics. Cell length was quantified from the distance between the upper and lower cell margin in the scanned line. In confocal line scans this distance represents the cell length in the confocal plane (“projected” cell length). In transmitted light line scans (Online Fig. V), the outer margins delineate the overall cell length.

The distance between adjacent sarcomeres (local sarcomere length) was measured in binary images derived from the Alexa 594 WGA line scan images following thresholding. Local sarcomere length was quantified at 5 regularly spaced time points between maximal local contraction and maximal local relengthening (Fig. 6D). The time constant of local relengthening (T_{AU_{local_RL}}) was calculated by mono-exponential curve fitting at 6±1 different positions along the scan-line and compared to T_{AU_{local}} of the [Ca^{2+}] transient simultaneously recorded at the same position.

TMRM images were converted into binary images using an automated algorithm coded in IDL. To account for small variations in the intensity of the stained mitochondria within the cell, the previously described algorithm was adapted. For each pixel, an individual threshold was calculated from the intensity histogram of the 5 µm x 5 µm neighborhood using the Otsu thresholding algorithm; only pixels within the cell margins were considered. As before, the signal threshold was calculated as the value resulting in maximal between-class variance of signal and background pixels in the defined neighborhood region. Calculated thresholds for each pixel resulted in a map of regional thresholds (Suppl Fig. 9 shows the threshold map for Online Fig. VIIIA, Sham). Pixel intensities of the original image were then thresholded by their respective threshold in the threshold map to obtain a binary image. High frequency noise was filtered by pre-assigned fixed binary erosion. Mitochondrial signal density was then quantified as the number of signal-positive pixels over all pixels within the cell margins.

Statistical analysis

Data are shown as mean±SEM. For comparison of T_{AU_{local}} in slowCaR and fastCaR (Figs. 3 and 4, Online Figs. I, II, IV, VI), Tau of slowCaR and fastCaR were averaged per cell and compared using a 2-
tailed Student’s t-test. Comparisons of TAU\textsubscript{global} and SD\textsubscript{TAU} in multiple groups (Fig. 3D, Fig 7, Online Fig. III) were performed by a one way ANOVA followed by a Bonferroni post-hoc test. TAU\textsubscript{global} was plotted as a function of SD\textsubscript{global} for individual cells of each group and the relationships between TAU\textsubscript{global} and SD\textsubscript{global} (Figs. 3E, 4D, 5B, 7A-B, Online Fig. II-V) were compared between groups by multiple linear regression analysis. P<0.05 was considered significant.

Supplementary Results

Effects of Istaroxime and Post-rest Potentiation on Regions of Slow and Fast Ca removal

With forskolin, we observed a stronger accelerating effect on cytosolic Ca\textsuperscript{2+} removal (TAU\textsubscript{local}) in slowCaR vs. fastCaR (see Results). A variety of signaling pathways may eventually be triggered by enhanced adenylyl cyclase (AC) activity with forskolin, even though it has been suggested that beta-adrenergic (AC mediated) stimulation of the cytosolic [Ca\textsuperscript{2+}] transient decay is entirely phospholamban and thus SERCA dependent.\textsuperscript{9} We used istaroxime, a Na\textsuperscript{+}/K\textsuperscript{+} ATPase inhibitor and direct SERCA stimulator\textsuperscript{8} to stimulate SERCA (1 µmol/L for 10 min, 1 Hz). Istaroxime significantly increased the [Ca\textsuperscript{2+}] transient amplitude (Online Fig. IV A; F/F\textsubscript{0}: 4.2±0.2 vs. 3.6±0.3 at baseline; n= 15 cells; P<0.05) and accelerated TAU\textsubscript{global} (166±17 vs. 283±43 ms at baseline; n= 15 cells; P<0.05).

In human non-failing cardiomyocytes (n=9 cells from 2 hearts), we measured the [Ca\textsuperscript{2+}] transient at 0.5 Hz steady state and the first electrically evoked [Ca\textsuperscript{2+}] transient after a stimulation pause of 10 seconds. In cardiomyocytes with post-rest potentiation, i.e. a higher peak [Ca\textsuperscript{2+}] transient amplitude (on average 32% higher than at steady state) and faster global [Ca\textsuperscript{2+}] decay reflecting increased SERCA activity (n=4 cells), [Ca\textsuperscript{2+}] decay in slowCaR was more accelerated that in fastCaR (Online Fig. IV C).

Variation in the rate of cytosolic [Ca\textsuperscript{2+}] decay was unchanged with altered NCX activity

The effect of inhibition of the sarcolemmal NCX with SEA0400 on local [Ca\textsuperscript{2+}] decay kinetics was evaluated in murine cardiomyocytes (Online Fig. II A). With the addition of SEA0400, TAU\textsubscript{global} (295±30 vs. 268±27 ms) and SD\textsubscript{TAU} (57±8 ms vs. 42±6 vs) were significantly increased. TAU\textsubscript{local} was similarly prolonged in fastCaR and slowCaR (108±1 and 112±1 % of baseline; n=5 cells), so that the relationship between TAU\textsubscript{global} and SD\textsubscript{TAU} was unchanged (Online Fig. II B). We confirmed these findings in the TAC mouse model which showed increased NCX1 protein expression and forward mode activity (Online Fig. III). In this model, NCX1 protein expression is increased more than two-fold already three weeks following TAC as confirmed by Western blot (NCX/GAPDH 2.32±0.26 vs. 0.98±0.09 in Sham; N=4 animals per group; P<0.05). Caffeine-induced [Ca\textsuperscript{2+}] transients showed a faster decline (TAU\textsubscript{caff}) in TAC (6 wks) vs. Sham (979 ± 83 vs. 2865 ± 517 ms; Online Fig. III A,B; n=5/5/7 for Sham/TAC/TAC+SEA0400 respectively), indicating increased NCX1 forward mode activity in TAC. As calculated from TAU\textsubscript{caff} and TAU\textsubscript{global} at steady state\textsuperscript{10}, contribution of NCX1 to cytosolic
[Ca\textsuperscript{2+}] removal was also increased during electrically stimulated [Ca\textsuperscript{2+}] transients (Online Fig. III C). In TAC, the NCX1-inhibitor SEA0400 (300 µmol/L) significantly decreased NCX1 forward mode activity (Online Fig. III A-C). However, the relationship between TAU\textsubscript{global} and SD\textsubscript{TAU} was not different in TAC in the absence and presence of SEA0400. Thus, also in this model with increased NCX activity, NCX inhibition with SEA0400 had no effect on the relationship between TAU\textsubscript{global} and SD\textsubscript{TAU}.

We compared the kinetics of local cytosolic [Ca\textsuperscript{2+}] decay in NCX1\textsuperscript{−/−} mice with their WT littermates (Fig. 4C). Surprisingly, TAU\textsubscript{global} was significantly shorter in NCX1\textsuperscript{−/−} vs. WT (185±36 vs. 307±80 ms; n≥15 cells/group). SD\textsubscript{TAU} was similarly decreased in NCX1\textsuperscript{−/−} (21±4 vs. 37±9 ms), reflecting an unaltered relationship between TAU\textsubscript{global} and SD\textsubscript{TAU} in NCX1\textsuperscript{−/−} vs. WT (Fig.4D).

Relation of cytosolic [Ca\textsuperscript{2+}] decay to sarcomere structure

We investigated whether the spatial differences in local Ca\textsuperscript{2+} removal kinetics may result in heterogeneity of Ca\textsuperscript{2+} removal along the same myofibril. To address this, we used Alexa594-staining of the T-tubules to define the sarcomere margins in Fluo-4 AM loaded murine cardiomyocytes (Online Fig. VII A). We calculated the local transients and TAU\textsubscript{local} from a sample width of 1 pixel (0.2 µm) along the scan line. Online Fig. VII C shows TAU\textsubscript{local} as a function of the relative position of the local transient within the half-sarcomere in 15 adjacent half-sarcomeres of a porcine cardiomyocyte (every symbol=1 half-sarcomere). TAU\textsubscript{local} of the outmost half-sarcomeres are marked by triangle symbols, the half-sarcomere in the center of the scan line section by a bold empty circle (see also Online Fig. VII A). The filled grey circles in Online Fig. VII C indicate the mean TAU\textsubscript{local} at a given position within the half-sarcomeres. As shown, mean TAU\textsubscript{local} did not depend on the position within the sarcomere. On the other hand, there was considerable variation in TAU\textsubscript{local} comparing a given position (e.g. 0.0 µm = at the Z-line/T-tubule) between the 15 adjacent half-sarcomeres (in this example with a range between 76% and 117% of TAU\textsubscript{global}). Additionally, the relation of TAU\textsubscript{local} between the 15 half-sarcomeres (i.e. the sequence of the symbols) varied at different positions within the half-sarcomere, indicating that the pattern of [Ca\textsuperscript{2+}] decay within a half-sarcomere was not uniform between adjacent half-sarcomeres. These observations suggest heterogeneity of Ca\textsuperscript{2+} removal between sarcomeres along the same myofibril.

Dyssynchrony in cytosolic [Ca\textsuperscript{2+}] decay is increased in porcine chronic ischemic myocardium

In a porcine model of chronic myocardial ischemia, cell length (129±1.4 vs. 123±1.6 µm; P<0.05) reflected mild cardiomyocyte hypertrophy in the infarct border zone \textsuperscript{5} (CTRL: 19 hearts; n=57 cells; MI: 14 hearts; n=57 cells). SD\textsubscript{TAU} was significantly larger in cardiomyocytes from the infarct border zone as compared to cardiomyocytes from control animals (59±3 vs. 48±2 ms) whereas TAU\textsubscript{global} was comparable (Suppl Fig. 6). Similar to our findings in the mouse, the addition of forskolin accelerated cytosolic [Ca\textsuperscript{2+}] decay significantly more in slowCaR as compared to fastCaR (reduction of TAU\textsubscript{local} to 46±2% of baseline in slowCaR vs. 56±2% of baseline in fastCaR; n=10 cells, P<0.05).
Mitochondrial remodeling and dyssynchronous $\text{Ca}^{2+}$ removal

We found an increased mitochondrial signal density in non-failing human as compared to murine cardiomyocytes (Online Fig. VIII B, $N=11$ cells/group, $34.9\pm1$ Mouse vs. $38.9\pm0.6\%$ in Human). In TAC mice, the mitochondrial signal density was significantly higher than in SHAM and correlated with increased dyssynchrony of $\text{Ca}^{2+}$ reuptake (Online Fig. VIII C, $N=20$ TAC and $11$ SHAM cells $34.9\pm1$ SHAM vs. $38.1\pm0.7\%$ TAC).

Extended Discussion

In cardiac myocytes, coordinated $\text{Ca}^{2+}$ release from the SR determines cellular contraction. CICR is regulated locally in subcellular micro-domains. Recent evidence has shown that disruption of this intracellular organization, e.g. by the loss of T-tubules, contributes to contractile dysfunction in chronic ischemia, myocardial hypertrophy and heart failure.

We provide the first quantitative evidence for regional differences in the subcellular regulation of $[\text{Ca}^{2+}]$ decay in the cytosol of cardiac myocytes during diastole. The rate of $[\text{Ca}^{2+}]$ decay substantially varies between adjacent regions within the myocyte, and responds differentially to pharmacological modulation. Intracellular variation in the rate of cytosolic $[\text{Ca}^{2+}]$ decay was significantly increased in animal models of myocardial hypertrophy (following TAC) and chronic myocardial ischemia. Dyssynchrony was also significantly increased in end-stage human heart failure, associated with slowed global $[\text{Ca}^{2+}]$ decay.

In the present study, we used the standard deviation ($\text{SD}_{\text{TAU}}$) as an indicator of spatial variability in the rate of cytosolic $[\text{Ca}^{2+}]$ decay. It seems reasonable to interpret $\text{SD}_{\text{TAU}}$ in the context of mean TAU ($\text{TAU}_{\text{global}}$). Indeed, we found a positive linear relationship between these two parameters in cardiomyocytes under similar conditions. However, interventions and conditions leading to altered intracellular $\text{Ca}^{2+}$ trafficking had differential effects on $\text{SD}_{\text{TAU}}$ and $\text{TAU}_{\text{global}}$, resulting in significant alterations in their relationship and confirming $\text{SD}_{\text{TAU}}$ and $\text{TAU}_{\text{global}}$ as two complementary parameters in the regulation of cytosolic $[\text{Ca}^{2+}]$ decay.

A variety of processes are regulated by local cytosolic $[\text{Ca}^{2+}]$, and different rates of cytosolic $[\text{Ca}^{2+}]$ decay may result in dyssynchrony of $\text{Ca}^{2+}$-mediated processes during the cardiac cycle, as we have documented with local sarcomere relengthening. In addition to the rate of local $[\text{Ca}^{2+}]$ decay, dyssynchrony may be influenced by variation in the onset of local $\text{Ca}^{2+}$ removal which is determined by the onset of $\text{Ca}^{2+}$ release into the cytosol. We demonstrate in Fig. 5C that the onset of local $\text{Ca}^{2+}$ release significantly influences the timing of restoration of cytosolic $[\text{Ca}^{2+}]$ ($\text{RT}_{90}$), but not the rate of $[\text{Ca}^{2+}]$ decay. Therefore, the onset of $\text{Ca}^{2+}$ release (and thus removal) and the rate of cytosolic $[\text{Ca}^{2+}]$ decay are independent determinants of dyssynchrony (Fig. 5C).
SR Ca\(^{2+}\) reuptake via SERCA and NCX1-mediated Ca\(^{2+}\) extrusion are the main pathways of Ca\(^{2+}\) removal from the cytosol in cardiac myocytes. Sarcolemmal NCX1 is closely associated with phosphatases, kinases, and regulatory proteins, most importantly phospholemman \(^{14}\), which form a potential basis for local regulation. NCX1 is distributed along the sarcolemmal membrane and the T-tubules. We have shown previously in pig cardiomyocytes that regions with early and delayed Ca\(^{2+}\) release, reflect regions with high and low T-tubule density, respectively \(^{1,5}\). In the present study, the kinetics of Ca\(^{2+}\) release was not related to the kinetics of local [Ca\(^{2+}\)] decay, suggesting that the distribution of T-tubules as a scaffold for NCX1 did not have a large influence on the regional variation in the rate of local cytosolic [Ca\(^{2+}\)] decay in healthy cardiomyocytes. Furthermore, our findings of similarly prolonged Ca\(^{2+}\) decay in all cell regions with NCX-inhibition (in healthy and also in TAC mice with higher NCX activity) and the unaltered variability of Ca\(^{2+}\) decay kinetics in NCX1 heterozygous mice argue against a role for the sarcolemmal NCX in the observed intracellular variation of the cytosolic [Ca\(^{2+}\)] decay rate. However, as NCX1 activity in human myocardium has been reported to contribute to an even larger extend to cytosolic Ca\(^{2+}\) removal \(^{15}\), the role of NCX for dyssynchrony in human myocardium remains to be investigated.

Dyssynchronous Ca\(^{2+}\) removal could potentially be explained by an inhomogenous distribution of SERCA2a protein along the SR. Our experiments in intact primary cardiomyocytes did not allow for simultaneous visualization of SERCA distribution. However, previous reports indicate a homogenous sarcomeric distribution pattern of SERCA2a \(^{16,17}\). SERCA activity is also modulated by protein-protein interaction, with phospholamban as the pivotal inhibitory regulator. However, it recently became evident that SERCA also forms a macromolecular protein complex with other regulatory proteins (e.g. calreticulin, S100A, and others; see \(^{18}\) for a review), and is also regulated by direct biochemical modification \(^{19}\) and local metabolic processes \(^{20}\). In the present study, local cytosolic [Ca\(^{2+}\)] decay in slowCaR was significantly more sensitive to SERCA stimulation and inhibition (Fig. 3, Online Fig. IV), indicating a larger role of SERCA in removing cytosolic Ca\(^{2+}\) in slowCaR regions vs. fastCaR regions. The rate of SR Ca\(^{2+}\) reuptake by SERCA depends on cytosolic [Ca\(^{2+}\)] \(^{21}\), which could imply that local Ca\(^{2+}\) release may affect the kinetics of local SR Ca\(^{2+}\) reuptake by SERCA. However, neither the timing of local Ca\(^{2+}\) release (Fig. 5C) nor the local peak amplitude of cytosolic [Ca\(^{2+}\)] (Fig. 2A) contributed significantly to the observed variance in the local kinetics of cytosolic [Ca\(^{2+}\)] decay.

In the present study we observed only little beat-to-beat variability in the distribution of slowCaR and fastCaR during steady state stimulation. In addition, faster Ca\(^{2+}\) turnover with higher stimulation frequency (Fig. 5B) increased the spatial variability in TAU\(_{\text{local}}\). These observations may indicate that the variability in TAU\(_{\text{local}}\) is related to the distribution of intracellular structures. We found that regions of fast [Ca\(^{2+}\)] decay were more often associated with regions of increased mitochondrial signal density (Fig. 4). The role of mitochondrial Ca\(^{2+}\) uptake in regulating ECC is not completely understood. Several \(^{22-24}\) but not all studies (see \(^{25}\) for review) found that mitochondrial Ca\(^{2+}\) uptake occurs on a beat-to-beat basis and may affect cytosolic [Ca\(^{2+}\)] transients \(^{22,23}\). Mitochondrial Ca\(^{2+}\) uptake is facilitated by high Ca\(^{2+}\) gradients as they occur when mitochondria and the Ca\(^{2+}\)-releasing SR are in close proximity \(^{26,27}\). Mitochondria and the endoplasmatic reticulum (ER) form narrow
compartments, which are constituted by direct links between the ER membrane and the outer mitochondrial membrane (“tethers”), and possibly by functional coupling via macromolecular complexes. SERCA is sensitive to mitochondrial ATP production and the organization of mitochondria and SR may directly influence the rate of SR Ca\textsuperscript{2+} reuptake. However, the role of metabolic regulation for subcellular differences in cytosolic [Ca\textsuperscript{2+}] decay remains to be determined.

As the time course of cytosolic [Ca\textsuperscript{2+}] decay correlates with cardiomyocyte relaxation (Fig. 6A,B), we hypothesized that local variation in the decay of cytosolic [Ca\textsuperscript{2+}] may be associated with different rates of sarcomere relengthening within the same cardiomyocyte, as confirmed in Fig. 6E. Interestingly, several authors have shown before, that sarcomere relaxation is not uniform in striated muscle (see for review). Edman et al. hypothesized that non-uniform changes in sarcomere lengths are mainly due to regional differences in the rate of Ca\textsuperscript{2+} removal. In the present study, we provide the evidence for regional inhomogeneities in [Ca\textsuperscript{2+}] decay giving rise to dyssynchronous sarcomere relengthening.

Dyssynchronous sarcomere relengthening can influence the kinetics of muscle tension decay. Stern et al. suggested that local diastolic Ca\textsuperscript{2+} gradients (related to spontaneous Ca\textsuperscript{2+} waves) may impair relaxation by increasing diastolic tension, as well as reducing contraction during the subsequent beat. Slow decay of [Ca\textsuperscript{2+}] from the cytosol may reactivate cross-bridge cycling, and it is conceivable that the subcellular synchrony of cytosolic [Ca\textsuperscript{2+}] decay and sarcomere relengthening determines the efficacy of cardiomyocyte relaxation.

In multicellular preparations and at increased mechanical load myofilament cross-bridge kinetics are the major rate determining step in relaxation. As our recordings were performed in isolated unloaded cardiac myocytes, the correlation between parameters of intracellular [Ca] decay (i.e. TAU\textsubscript{global} or SD\textsubscript{Tau}) and the rate of relaxation (Online Fig. V D) thus may be confounded in vivo by slower myofilament relaxation. The role of dyssynchronous Ca\textsuperscript{2+} reuptake for mechanical relaxation in vivo therefore remains to be determined. On the other hand, cytosolic Ca\textsuperscript{2+} removal is the initiating step for relaxation and the rate of cytosolic Ca\textsuperscript{2+} removal does influence relaxation kinetics in isolated cells, multicellular preparations and also in vivo. Interestingly, if Ca\textsuperscript{2+} is removed (nearly) instantaneously, mechanical non-uniformity in relengthening of sequential sarcomeres may even facilitate myofilament relaxation. The spatial differences in local Ca\textsuperscript{2+} removal kinetics we describe here likely also result in heterogeneity of Ca\textsuperscript{2+} removal of contiguous sarcomeres of the same myofibril (exemplified in Online Fig. VII). However, even though some extent of dyssynchrony may provide a benefit for relaxation in physiological conditions, we clearly show that increased dyssynchrony is associated with slowed Ca\textsuperscript{2+} decay and cardiomyocyte relaxation. Additionally, passive stretching of weaker (“yielding”) sarcomeres by their contracted neighbors results in reduced myofilament Ca\textsuperscript{2+} binding, and Ca\textsuperscript{2+} surges released from the myofilaments during non-uniform contraction have been identified as a cause of arrhythmias. Thus, an increased dyssynchrony in intracellular [Ca\textsuperscript{2+}] decay and sarcomere relengthening may in several ways impair cardiomyocyte function.
A significantly increased level of dyssynchrony in cytosolic \( \text{Ca}^{2+} \) decay was observed in LV cardiomyocytes from murine hypertrophied failing hearts following TAC (Fig. 7A), associated with slowed cardiomyocyte relengthening in TAC (Online Fig. V C). Similarly, in the porcine model of chronic regional myocardial ischemia, a significant, albeit less pronounced, increase in dyssynchrony was observed in cardiomyocytes from the infarct border zone. Finally, we could confirm in human hearts, that an elevated level of dyssynchrony is characteristic for stage D\(^{46}\) heart failure.

Both models and human end-stage heart failure are associated with cardiomyocyte hypertrophy as a result of myocardial remodeling (Fig. 7C,\(^8\)). Although confocally derived cell length in one plane is only an estimate of total cell size, our data indicates that dyssynchrony in cytosolic \( \text{Ca}^{2+} \) decay is related to cellular hypertrophy.

In electron micrographs mitochondrial density is higher in human as compared to rodent hearts\(^49\). Similarly, we found an increased mitochondrial signal density in non-failing human as compared to murine cardiomyocytes (Online Fig. VIII). In TAC mice, mitochondrial signal density was significantly higher than in Sham and correlated with increased dyssynchrony of \( \text{Ca}^{2+} \) reuptake. Mitochondrial alterations in cardiac remodeling have been described before, with an increased mitochondrial mass reported in some\(^{45-47}\) but also a decrease in mitochondrial size (mitochondrial fission) observed in other models\(^48\). An increase in mitochondrial volume indicating mitochondrial remodeling was also found in electron micrographs in the present TAC model\(^15\). Despite this evidence, it is currently unclear why mitochondrial remodeling with increased mitochondrial density is associated with more dyssynchrony, and we cannot exclude that additional mechanisms leading to heterogeneity in local mitochondrial or SR function contribute to dyssynchronous cytosolic \( \text{Ca}^{2+} \) removal.

Interestingly, dyssynchrony in \( \text{Ca}^{2+} \) removal was not increased in the hearts from donors with no clinical history of heart failure but adverse LV remodeling and mild to moderately reduced EF, equivalent to heart failure stage B\(^{46}\). As global LV function was also only mildly reduced in the porcine model\(^5\), our results highlight the close association between regional myocardial function and cellular function of the cardiomyocytes.

In summary, we show that dyssynchronous diastolic \( \text{Ca}^{2+} \) decay within individual cardiomyocytes contributes to dyssynchronous intracellular sarcomere relengthening. Intracellular dyssynchrony of \( \text{Ca}^{2+} \) decay is increased in animal models and in human heart failure and represents a potential new mechanism underlying contractile dysfunction in cardiac remodeling.
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**Online Table I: Clinical characteristics of the human heart samples.** Hearts were divided in 3 groups: non-failing donor hearts with preserved ejection fraction, donor hearts without clinical history of heart failure (HF) but with echocardiographic signs of remodeling and reduced ejection fraction, and explanted end-stage failing hearts. LVEDD, left ventricular (LV) end-diastolic diameter; LVSED, LV septum end-diastolic thickness; LVPWed, LV posterior wall end-diastolic thickness; LVEF, LV ejection fraction; PAPsys, systolic pulmonary artery pressure, as derived from the tricuspidal regurgitation Doppler signal. *P<0.05 vs. non failing; #P<0.05 vs. remodeling.
Online Figure I. SERCA modulation predominantly affects regions of slow [Ca\textsuperscript{2+}] decay.  

A. Line scan image of murine cardiomyocyte before (top) and in presence (middle) of SERCA inhibitor CPA. Red line: Distribution of TAU\textsubscript{local}. TAU\textsubscript{local} was significantly more prolonged in slow (slowCaR) vs. fast (fastCaR) [Ca\textsuperscript{2+}] decay regions (See Fig. 3D, bottom; *P<0.05, n=7 cells). B. Examples of local [Ca\textsuperscript{2+}] transients showing a stronger effect of CPA in regions of slow (slowCaR) vs. fast (fastCaR). C. Local [Ca\textsuperscript{2+}] transients normalized to the peak amplitude further emphazise the dysproportional increased effect of CPA on slowCaR.
Online Figure II. NCX activity does not affect dyssynchrony in local $[Ca^{2+}]$ decay. A. Line scan image of murine cardiomyocyte before (left) and in the presence of (right) the NCX inhibitor SEA0400. Red line: Distribution of TAU$_{local}$. B. Mean values of TAU$_{local}$ and SDTAU were similarly affected by SEA0400. C. Linescan images of WT (left) and NCX1 heterozygous (NCX1+/-, right) mice. D. The relationship between TAU$_{global}$ and SDtau is similar in WT and NCX1+/- (n= 26 cells for NCX+/-, n=15 for WT).
Online Figure III. NCX activity does not affect dyssynchrony in local [Ca^{2+}] decay in mice with TAC-induced heart failure. A. Electrically stimulated [Ca^{2+}] transients followed by the application of caffeine (20mM) in Sham (top) and TAC (middle) mice, and in TAC mice treated with SEA0400 (bottom; 0.3 µmol/L; n=5/5/7 cells resp. from 3 Sham and 3 TAC hearts). B. Decay of the caffeine-induced [Ca^{2+}] transient was significantly accelerated in TAC vs. Sham (upper panel; *P<0.05; +P=0.05); the amplitude (F_peak) of global [Ca^{2+}] transients at steady state (1 Hz) was not significantly affected (lower panel). The contribution of NCX to Ca^{2+} removal at 1 Hz steady state was increased in TAC (*P<0.05 vs. CTRL) and reduced to CTRL level in TAC+SEA0400 (#P<0.05 vs. TAC; see text for details). D. The relationship between TAU_{global} and SD_{TAU} is not significantly changed before and after SEA treatment (n=11 TAC cells from 3 hearts).
Online Figure IV. Different types of SERCA modulation predominantly affect regions of slow \([Ca^{2+}]\) decay. **A.** Cytosolic example \([Ca^{2+}]\) transients (1 Hz electrical stimulation, left) at baseline (black) and after addition of Istaroxime (red) to the cell bath. Istaroxime predominantly affects slowCaR (center; N=3 mouse hearts, n=15 cells, *p<0.05). The linear correlation between TAU\(_{\text{global}}\) and SD\(_{\text{TAU}}\) at baseline is altered by istaroxime and reflects significantly less variability in local \([Ca^{2+}]\) decay kinetics as measured by SD\(_{\text{TAU}}\) (right). **B.** Line scan image of murine cardiomyocyte before (left) and in the presence of (right) the SERCA activator istaroxime (1 µmol/L). Red line: Distribution of TAU\(_{\text{local}}\). **C.** Cytosolic example \([Ca^{2+}]\) transients (0.5 Hz electrical stimulation, arrows) following several seconds without stimulation recorded in a non-failing human ventricular cardiomyocyte (left). 4/9 cardiomyocytes (N= 2 hearts) showed an increased \([Ca^{2+}]\) transient amplitude and a faster \([Ca^{2+}]\) decay at the first beat post rest indicating post rest potentiation. In this subset of cells TAU\(_{\text{local}}\) in slowCaR was significantly more decreased than fastCaR (right; *p<0.05).
Online Figure V. Cardiomyocyte shortening and \([\text{Ca}^{2+}]\) decay in SHAM and TAC mice. A. Transmitted light line scan of a SHAM and a TAC cardiomyocyte and corresponding cell length recordings (B) at steady state (1 Hz; SHAM=black; TAC=grey). C. Time to peak shortening and \(\text{TAU}_{\text{global}}\) relengthening are significantly increased in TAC (n=18 cells for SHAM, n=35 cells for TAC; 122±9 vs. 80±4 ms in SHAM, 71±7 vs. 19±1 ms in SHAM, resp., *p<0.05). D. \(\text{TAU}_{\text{global}}\) of cell relengthening in SHAM and TAC increased with \(\text{TAU}_{\text{global}}\) of the \([\text{Ca}^{2+}]\) transient (left). \(\text{TAU}_{\text{global}}\) of the \([\text{Ca}^{2+}]\) transient tended to increase with \(\text{SD}_{\text{TAU}}\) in TAC (right; n=6 cells from 2 hearts per group).
Online Figure VI. Pig, chronic myocardial ischemia. Example line scan images (top,left) of cardiomyocytes from the infarct border zone (MI) and matching region in sham-operated animals (SHAM). SD$_{TAU}$ was increased (top,right;*P<0.05) and the linear relation between TAU$_{global}$ and SD$_{TAU}$ (detail) was significantly altered in MI vs. Sham. Bottom, left: SD$_{TAU}$ increased with cell length (P=0.055 for lin.regression). Bottom, right: In MI, forskolin shortened TAU$_{local}$ significantly more in slowCaR. *P<0.05 vs. fastCaR, n=10 cells/group. #P<0.05 vs. MI-Pig baseline, n=10 cells/group.
Online Figure VII. No significant \([\text{Ca}^{2+}]\) gradients within single sarcomeres during cell relaxation. **A.** Simultaneous recording of T-Tubules and local \([\text{Ca}^{2+}]\) transients in a pig ventricular myocyte. The black line depicts the signal intensity of structures stained with WGA-Alexa (presumably T-Tubules); Red line: Distribution of TAU\(_{\text{local}}\). The top, middle and bottom T-Tubules are marked separately (*Triangles and circle; See also Online Fig. 7C*). **B.** TAU\(_{\text{local}}\) was measured for local \([\text{Ca}^{2+}]\) transients at 0.2 µm intervals from the T-Tubule. **C.** TAU\(_{\text{local}}\) at the point of maximal WGA-Alexa intensity and at 0.2 µm distance intervals from the T-Tubule is not different. The triangles and circle mark specific T-Tubules (*See also Online Fig. 7A*).
Online Figure VIII. Mitochondrial remodeling and dyssynchronous Ca\(^{2+}\) removal. A. Top: Examples of mitochondrial signal (TMRM) in cardiomyocytes from SHAM and TAC. Below: Binary image of mitochondrial signal after automatic thresholding (see text for details; thin red line indicates cell margins; red region: nucleus was excluded from analysis); see also enlarged images below. B. Higher mitochondrial density in human (red) vs. murine (black) cardiomyocytes (right, n=11 cells/species) is associated with higher dyssynchrony in cytosolic Ca\(^{2+}\) removal (SD\(_{TAU}\), left, n=8 cells/species). C: In TAC mice, mitochondrial signal density was increased (right) and correlated with SD\(_{TAU}\) (left; n=20 cells/N=2 animals in TAC and n=11/N=2 in Sham).
Online Figure IX. Thresholding of Mitochondrial Signal. A. Top: Murine cardiomyocyte stained with TMRM (from Fig. 8). Middle: Threshold Map. The calculated threshold for each pixel is coded as a grey-scale intensity. Note that higher intensity of the stained mitochondria at the left and right cell edges (top image) is compensated for by an increased threshold value in these regions. Bottom: Binary image of mitochondrial signal after automatic thresholding (see text for details; thin red line indicates cell margins; red region: nucleus was excluded from analysis).
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