Microdomain-Specific Modulation of L-type Calcium Channels Leads to Triggered Ventricular Arrhythmia in Heart Failure

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Running title: L-type Calcium Channel Relocation in Heart Failure

Subject Terms:
Electrophysiology
Heart Failure
Imaging
Ion Channels/Membrane Transport
Translational Studies

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In July 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.27 days.
ABSTRACT

**Rationale:** Disruption in subcellular targeting of Ca^{2+} signaling complexes secondary to changes in cardiac myocyte structure may contribute to the pathophysiology of a variety of cardiac diseases, including heart failure (HF) and certain arrhythmias.

**Objective:** To explore microdomain-targeted remodeling of ventricular L-type Ca^{2+} channels (LTCCs) in HF.

**Methods and Results:** Super-resolution scanning patch-clamp, confocal and fluorescence microscopy were used to explore distribution of single LTCCs in different membrane microdomains of non-failing and failing human and rat ventricular myocytes. Disruption of membrane structure in both species led to re-distribution of functional LTCCs from their canonical location in transversal tubules (T-tubules) to the non-native crest of the sarcolemma, where their open probability (P_o) was dramatically increased (0.034±0.011 vs 0.154±0.027, P<0.001). High P_o was linked to enhanced calcium-calmodulin kinase II (CaMKII)-mediated phosphorylation in non-native microdomains and resulted in an elevated $I_{Ca,L}$ window current which contributed to the development of early afterdepolarizations (EADs). A novel model of LTCC function in HF was developed; following its validation with experimental data, the model was used to ascertain how HF–induced T-tubule loss led to altered LTCC function and EADs. The HF myocyte model was then implemented in a 3D left ventricle model, demonstrating that such EADs can propagate and initiate reentrant arrhythmias.

**Conclusion:** Microdomain-targeted remodeling of LTCC properties is an important event in pathways that may contribute to ventricular arrhythmogenesis in the settings of HF-associated remodeling. This extends beyond the classical concept of electrical remodeling in HF and adds a new dimension to cardiovascular disease.

**Keywords:**
L-type calcium channel, microdomain, super-resolution scanning patch-clamp, heart failure, modeling.

**Nonstandard Abbreviations and Acronyms:**
HF   heart failure
LTCC   L-type Ca^{2+} channels
RyRs   ryanodine receptors
CaMKII   calcium/calmodulin kinase II
MI   myocardial infarction
SICM   scanning ion conductance microscopy
TT   T-tubule
EADs   early afterdepolarizations
AP   action potential
P_o   open probability
INTRODUCTION

Heart failure (HF) is a major cause of morbidity and mortality, contributing significantly to global health expenditure. Sudden death due to arrhythmia is responsible for over 50% of deaths among HF patients and therefore preventing arrhythmia and ameliorating the risk of sudden cardiac death secondary to HF is a paramount clinical need. Pathophysiological remodeling of cardiac function in HF occurs at multiple levels and includes the alterations in ion channel profile, Ca\(^{2+}\)-handling proteins, and proteins mediating cell-cell coupling, predisposing to arrhythmias. Recent studies have demonstrated that disruption of proper cellular organization accompanied by a progressive loss of transverse tubule (T-tubule, TT) microdomains in HF, may also have an impact on calcium cycling thus promoting the development of arrhythmogenic triggers. Specifically, it has been hypothesized that the communication between L-type Ca\(^{2+}\) channels (LTCCs) and ryanodine receptors (RyRs) is impaired in HF, perhaps indicating that LTCCs are less strictly confined to TTs. In addition, LTCC protein phosphorylation defects have been identified in HF. Therefore, we hypothesized that there is a altered distribution of single LTCCs in cellular microdomains in HF following TT degradation, which results in channel dysfunction critically contributing to the development of arrhythmogenic triggers.

Here, we used the super-resolution scanning patch-clamp technique to probe the microdomain-specific localization of functional LTCCs with nano-spatial resolution in normal and failing ventricular cardiac myocytes. We showed, in failing cells, dislocation of functional LTCCs to the sarcolemma surface (or “crest” membrane, a term based on micrograph topography in contrast to TT), where they are rarely present in healthy cardiac myocytes. Obtaining evidence from multiple imaging modalities, electrophysiology and biochemistry, we discovered that these relocated channels exhibit higher open probability (P\(_{o}\)) and phosphorylation status, which we found to be linked to enhanced activity of calcium-calmodulin kinase II (CaMKII). The experiments were complemented by the development and use of a novel accurate HF computational model which also includes single channel behaviour to ascertain that LTCC relocation to the cell crest combined with enhanced CaMKII activity gives rise to the measured P\(_{o}\) values. The model then demonstrated how this abnormal behaviour leads to cell-level oscillations in membrane voltage and development of arrhythmogenic triggers, and how these propagate to become arrhythmias at the organ level. The combined experimental/simulation approach presented here provides a comprehensive understanding of how disease-induced remodelling at the microdomain level is manifested into dysfunction at the organ level.
METHODS

For full details of methods, please see the Online Supplement.

Study approval.
All animal experiments were carried out in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, incorporating the EU Directive 2010/63/EU, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Experiments on isolated human cardiac myocytes were approved by the Imperial College Institutional Review Board.

Myocytes isolation and T-tubule characterization.
Failing ventricular myocytes were isolated from 16-weeks post-MI rat model of HF (Online Figure I) and transplanted human hearts from patients with dilated cardiomyopathy (Online Table I). Age-matched sham operated rats and tissue biopsy human samples (Online Table II) were used to isolate control, non-failing cardiac myocytes. The subcellular TT system was visualized by confocal imaging of Di-8-ANEPPS stained cells. Surface topography was characterized by scanning ion conductance microscopy (SICM) which uses a glass nano-pipette as sensitive probe as described elsewhere.

Super-resolution scanning patch-clamp with pipette clipping modification.
After generating a topographical image of the cell surface by Scanning Ion Conductance Microscopy (SICM), the tip diameter of the pipette was widened by clipping to increase the area of attachment. The pipette was then lowered to a specific location until it touched the membrane and a high resistance seal was established. Single LTCC recordings were then performed in a cell-attached mode. Controlled widening of the scanning nano-pipette tip is described in detail in the Online Supplement (Online Figure II and Online Figure III).

Optical mapping of calcium activity.
Optical mapping of cells loaded with the Ca\textsuperscript{2+}-sensitive fluorescent dye Fluo-4AM via CMOS camera ULTIMA-L (SciMedia, USA Ltd., CA; 1,000 fps, 1.5-2 µm/pixel) was used to monitor localized changes in [Ca\textsuperscript{2+}]\textsubscript{i}.

Western blot.
Western blotting was done using monoclonal anti phospho-CaMKII (Thermo Scientific, MA1-047) α-tubulin (Sigma, T9026) and GAPDH (Santa Cruz Biotechnology, FL-335) primary antibodies, followed by secondary anti-mouse antibodies ECL detection. Western blots were analysed by using ImageJ software.

Statistical analysis.
All graphs and statistical analysis were performed using either GraphPad prism 5 or Origin version 6.1. Normality was tested using the Kolmogorov-Smirnov test. In cases where data failed the normality test, the nonparametric Mann-Whitney test was used instead of the unpaired Student t-test. Statistical differences were assessed with Student t-test, Mann-Whitney test, Kruskal-Wallis test and Fisher’s exact test as appropriate. All data are expressed as mean ± standard error of the mean (SEM). A value of P<0.05 was considered statistically significant.

Computational simulations.

Single Channel Human L-type Ca\textsuperscript{2+} Current (I_{Ca,L}): Human ventricular cell electrophysiological behavior was represented by the O’Hara-Rudy model. To model stochastic single channel behavior and determine the channel P_o for comparison with human experimental data, for I_{Ca,L}, we used its Markov-equivalent representation (Online Figure IV). To evolve the channel gating in response to a 1-s voltage change step...
from resting state to -6.7mV, we used the Gillespie Exact Algorithm. Barium simulations matched barium experiments; with this as validation, we extrapolated to physiological calcium simulations. Example single channel sweeps are shown in Online Figure V.

Once single channel current simulation results were generated and validated with experimental $P_o$ measurements, the $I_{Ca,L}$ model was reverted back to a Hodgkin-Huxley formulation, retaining the CaMKII mode definitions and the behavior of the equivalent Markov version. Failing crest LTCCs were assumed to operate in CaMKII phosphorylated mode, based on experimental findings (see Results); their inactivation was via the slow gating mode. Failing LTCCs in TTs and control LTCCs were sensitive to standard CaMKII, and so inactivation was both fast and slow. Ensemble current computed by summation of single channel sweeps matched the deterministic Hodgkin-Huxley current (Online Figure VI).

**Models of human control and failing myocytes.**

The original O'Hara-Rudy myocyte model did not include a sub-sarcolemmal volume and thus did not allow for Ca$^{2+}$ accumulation near the intracellular mouth of Crest LTCCs. We thus incorporated sub-sarcolemmal volume and related fluxes in the O'Hara-Rudy myocyte model based on the work of Grandi et al. and Shannon et al. Description of how this was done can be found in the Online Supplement.

The original O'Hara-Rudy model included LTCCs exclusively at TT sites with dyadic intracellular face. Here, LTCCs in TTs sensed and contributed to dyadic Ca$^{2+}$; channels newly added to the crest sensed and contributed to sub-sarcolemmal Ca$^{2+}$. In each of TT and crest locations, LTCC permeability, $P_Ca$, representing whole-cell current density in TTs ($P_{Ca_{TT}}$) and crest ($P_{Ca_{Crest}}$) needed to be defined. We utilized experimental data obtained in this study to assign values to $P_{Ca_{TT}}$ and $P_{Ca_{Crest}}$ in control and failing human cells. The data used were: % occurrence of LTCCs, LTCC single channel current amplitude, and in failing cells, the degree of TT loss. Specific values for $P_{Ca_{TT}}$ and $P_{Ca_{Crest}}$ in control and failing human cells and the methodology by which they were assigned can be found in the Online Supplement.

Outside of $I_{Ca,L}$ several factors were included to recreate an accurate HF model. Descriptions of the formulations for Na$^+$/Ca$^{2+}$ Exchanger (NCX), representation of “orphaned” RyRs, and additional parameters representing others HF ion channel remodeling in the myocyte model can be also be found in the Online Supplement.

**Whole heart model.**

Organ-level simulations were performed using an MRI-based, anatomically-realistic human left ventricular model described previously. Fiber orientation was assigned using a rule-based approach. A computational mesh at a resolution of ~300μm was generated using a validated approach. Transmural cell types and conductivity were specified according to experimental data from human left ventricles (Online Figure VII). All parameters defining differences between the endocardial and epicardial cell variations of the O'Hara-Rudy model were scaled linearly across the ventricular walls to generate smoothly varying intermediate transmural types (10 segments defined using a Laplace-Dirichlet technique). Pacing was delivered to the apex using a 2-cm diameter “electrode”. Electrophysiological simulation and numerical methods were identical to previous work. Simulations were executed in monodomain mode using the CARP software package.
RESULTS

HF is associated with loss of TTs with consequent alterations in LTCC spatial distribution and their functional properties.

In both rat and human failing myocytes, we observed a significant decrease in internal TT density as compared to non-failing controls (Fig.1A and B; ~50% decrease in human, P<0.01), consistent with our previous findings. Using SICM we found that the loss of TT correlates with the loss of surface structures as well (Fig.1C). Using Z-groove index as metric of surface integrity, we found that in both human and rat failing myocytes surface structure was impaired (Fig.1D, ~40% reduction in human, P<0.01). We also detected reduction in the number of TT openings on the surface of failing cardiac myocytes, identified as dark circles in SICM images (~35% reduction in rat compared with control, P<0.001; Online Figure VIII). The loss of TT microdomains in failing myocytes was accompanied by altered spatial distribution of LTCCs. In control rat and human cardiac myocytes, LTCC activity was predominantly recorded in TTs (26.7% of 86 successful patches in rat and 28.6% of 21 successful patches in human cardiac myocytes showed LTCC activity) as opposed to the crest, where LTCC activity was rarely recorded (only 7.02% of 57 successful patches in rat and 9.1% of 11 successful patches in human cells showed LTCC activity, Fig.2A and B, control). This confirmed our previous observation that the majority of functional LTCCs reside in the TTs. Interestingly, LTCC occurrence along the Z-groove in rat cardiac myocytes was found to be intermediate between that in TT and Crest areas (15% of 20 successful patches) suggesting a density gradient of channels throughout the membrane.

In contrast, in failing rat cardiac myocytes, LTCC current was recorded with similar frequency from both TTs and crests (20.4% of 54 successful patches in the TTs vs. 27.3% of 55 successful patches in the crest showed LTCC activity). In failing human cardiac myocytes, LTCC activity was even higher in the crest (27.3% of 22 successful patches in the TTs vs. 34.6% of 26 successful patches in the crest showed LTCC activity). This indicates that the distribution of functional LTCCs in the cardiac myocyte membrane was significantly altered in HF (Fig.2A and B, failing).

Our experiments demonstrated that the HF-associated increase in the number of functional LTCCs outside of their native microdomains is accompanied by changes in their behavior. The LTCC P_o was significantly elevated at the crest of failing myocytes as compared to that in crest of control myocytes in rat (P_o at -6.7mV: 0.034±0.011 for control crest LTCCs vs. 0.153±0.026 for failing crest LTCCs, P<0.001; Fig.2D) and to that in TTs (0.053±0.005 for control TT LTCCs, P<0.001; 0.051±0.008 for failing TT LTCCs, P<0.01; Fig.2D and F). No changes in LTCC amplitude were observed in rat HF (Online Figure IX). In failing humans myocytes the P_o was also significantly elevated at the crest as compared to that in TT of control and failing myocytes (P_o at -6.7mV: 0.136±0.025 for failing crest LTCCs vs. 0.043±0.01 for control TT LTCCs, P<0.01 and vs. 0.042±0.021 for failing TT LTCCs, P<0.05; Fig.2C and E). We next endeavored to determine the mechanisms for LTCC functional changes in HF.

Constitutive phosphorylation of LTCCs by cytoplasmic CaMKII leads to an increase in LTCC P_o.

Elevated phosphorylation can lead to increased P_o of LTCCs. Also, CaMKII activity is elevated in HF, and CaMKII can phosphorylated LTCCs at specific sites. Therefore, we tested the hypothesis that the elevated activity of CaMKII in failing cardiac myocytes is responsible for phosphorylation and thus for the high P_o of LTCCs in crest.

Western blots indicated that the phosphorylated CaMKII T286 was higher in rat and human failing cells (Fig.3A-B); this p-CaMKII could be reduced following the application of the CaMKII inhibitor KN-93 (Fig.3C; only rat myocytes were used in these experiments because of the paucity of human cells). As this residue is critical for the association and phosphorylation of the LTCC β2 subunit, these data indicate
that LTCC phosphorylation should be increased. Besides, as we have shown in Fig.3D and Online Figure X local \( P_o \) measurements demonstrate that LTCC \( P_o \) in the crest of failing myocytes was reduced to a value similar to control following the application of KN-93 (from 0.153±0.026 for failing crest LTCCs to 0.061±0.018 for failing crest LTCCs with KN-93, \( P<0.01 \)). LTCCs from control cells or from TT failing cells were not affected by the inhibitor (Fig.3D), suggesting that the increase of CaMKII in failing cells had only effect on the failing myocyte crest, confirming our hypothesis. It has been shown that KN-93 can block LTCC directly under certain conditions \(^{29} \); to strengthen our conclusion, AIP (Autocamptide-2 Related Inhibitor Peptide) was used on failing crest channels (Fig. 3B) and the results showed reduction of \( P_o \) to control values (0.031±0.003, \( P<0.05 \) vs failing crest values), confirming our initial findings.

Abnormal \([Ca^{2+}]]\) behavior in failing cells.

Since HF-remodeling altered LTCC function in failing myocytes, we investigated whether there were concomitant changes in \( Ca^{2+} \) transients. Optical mapping of \( Ca^{2+} \) transients at pacing rates of 0.5 Hz to 1 Hz revealed spontaneous \([Ca^{2+}]]\), oscillations which occurred during the decay phase in a greater proportion of failing cardiac myocytes compared to control (0.5Hz, \(~45\%) failing vs. \(~4\%) control \( P<0.05 \); Fig.4A). These \( Ca^{2+} \) oscillations were completely suppressed after treatment with KN-93 (Fig.4B) but not by KN-92 (Online Figure XI), an inactive analogue of KN-93. This suggests that the phosphorylation inhibition by KN-93 reduces the late LTCC current resulting from the increased LTCC \( P_o \) at the cell crest and prevents \([Ca^{2+}]]\) oscillations.

In rat failing cardiac myocytes, whole-cell patch clamp recordings (Online Figure XII) show prolonged action potential duration (APD) similar to what had been previously described in HF. \(^{30-31} \) It has been shown that such action potential duration prolongation is accompanied by spontaneous occurrence of single and multiple early afterdepolarizations (EADs) in failing ventricular myocytes. \(^{31} \) Oscillations in the \( Ca^{2+} \) transient such as shown in Fig.4, occurring in the settings of HF-associated ion channel remodeling, are also widely associated with EADs, a class of possible arrhythmogenic triggers in the heart. \(^{32} \) However, linking \( Ca^{2+} \) transient oscillations to the development of HF-related arrhythmias at the organ level is a challenging task, both in terms of demonstrating by experimentation, across the spatial scales of structural hierarchy, and also causally, since electrotonic influences at the tissue/organ level could suppress cell-level triggers. \(^{33} \) To prove that HF-induced changes in microdomain localization of LTCCs and their functional consequences at the cellular level could result in arrhythmia in the failing human heart, we developed and utilized a novel computational model of human HF that incorporated the experimental findings described above.

Computational model based on single channel kinetics predicts the development of cellular-level triggers of arrhythmias in human HF.

The human HF model was based on Monte Carlo simulations of the kinetics of a single human LTCC (see Online Supplement). Incorporating the experimental finding that in failing cardiac myocytes LTCCs at crests are CaMKII-phosphorylated, we calculated \( P_o \) values in TTs and crests in control and failing human myocytes (Fig.5A, example sweeps in Online Figure V). Simulations were able to relate LTCC phosphorylation at crests to the elevated local \( P_o \) value. The close match between simulation and experimental results served as a validation of the model, allowing us to use simulations to explore the downstream effect of LTCC functional changes.

Simulated human whole-cell \( Ca^{2+} \) current is of similar magnitude in failing and control cells, consistent with previous findings \(^{34-37} \) and our results on failing rat cardiac myocytes (Online Figure XIII). Current decay is slower in failing myocytes compared to control (Fig.5B), suggesting a potential for destabilization of repolarization. Slower decay is due to enhanced CaMKII phosphorylation of LTCC channels in the crest. Traces corresponding to TTs were nearly identical for failing and control cells with
regard to decay rate; however, TTs in failing myocytes were de-populated and thus current magnitude in TT was reduced relative to control.

The LTCC kinetics model was incorporated, together with other HF electrophysiological alterations, in an action potential (AP) model of the human (endocardial) ventricular myocyte to determine the cell-level consequences of LTCC dysfunction. AP simulation results showed that EADs developed due to late L-type Ca$^{2+}$ current appearance in failing myocytes only (Fig.6). Here, CaMKII was set to be maximal in the failing crest (validated by experimental data in Fig.5A), causing phosphorylation of all LTCCs there. CaMKII block eliminated L-type Ca$^{2+}$ current appearance and EADs. These simulation results were supported by optical [Ca$^{2+}$] recordings (Fig.4; Ca$^{2+}$ transient oscillations in failing human cells were simulated at a pacing rate of 0.25Hz, which could be considered similar to 0.5Hz pacing in the rat$^{15}$). Although EADs in isolated cells are potential ectopic triggers, their formation does not guarantee the occurrence of arrhythmogenic triggers or arrhythmias in the whole heart. We therefore conducted organ-level simulations to test whether EADs resulting from HF-induced LTCC re-localization could form propagating triggers and result in arrhythmia in the heart using more physiological human heart rates, such as 1Hz.

**Computational model predicts the development of arrhythmias in the failing human ventricles.**

Simulation results in Online Figure XIV demonstrated that epicardial cells did not develop EADs for any of the simulated conditions, indicating that arrhythmia triggers in the failing heart were expected to develop in the endocardial layer.

The formation of arrhythmogenic triggers and reentrant arrhythmia in the failing human heart is shown in Fig.7 (1Hz pacing with a single skipped beat; arrhythmia did not take place without a skipped beat at this pacing rate) and in the Online Video I. AP traces at 3 different endocardial locations are shown in Fig.7A; site “i” was the closest to the pacing location at the apex. In the control ventricles (Online Video II), wavefronts propagated in an organized fashion in response to each pacing stimulus (short horizontal gray lines relate stimuli, one-to-one, to resulting action potentials at sites throughout the ventricles). In the failing ventricles, EAD triggers appeared near site “ii” and propagated (dashed gray lines from EAD triggers towards triggered APs). Activation in the control ventricles was completed within 250 ms, undisturbed by the skipped beat (Fig.7B1 and B2). In contrast, activation took twice as long to excite the failing ventricles. Following the “skipped beat” pause, cells near site “ii” failed to repolarize, and an endocardial EAD trigger formed (Fig.7B1 and B2). As shown in Fig. 7C, triggered activity propagated from that location, resulting in reentrant arrhythmia. The skipped beat pause had no discernable effect on subsequent activity in the control model (results not shown). Simulations also showed that triggered activity and arrhythmias did not develop in the failing ventricles when CaMKII was blocked, or when crest LTCCs were made to sense dyadic [Ca$^{2+}$], where Ca-dependent inactivation was enhanced. The whole-heart simulations thus demonstrated a causal link between HF-induced microdomain localization of LTCCs and arrhythmias in the failing human heart.
DISCUSSION

This study adds a new dimension to the understanding of cardiovascular disease, highlighting microdomain-specific changes in LTCC function, which acts in concert with well-established changes in protein expression. The major discovery of this study is that a disruption in the delicately balanced dynamic interactions between LTCCs and their cellular microenvironment can lead to pathological changes in cellular physiology, and to a downstream dysfunction at the organ level. This novel concept may help to explain the molecular mechanisms of HF and other human diseases.

Re-localization of LTCCs in heart failure.

Here, for the first time, we provide direct evidence of the presence, in HF, of abnormally functioning LTCCs in the extradyadic space (crests) of ventricular cardiac myocytes, concurrent with changes in microdomain structure. These extradyadic LTCCs may lose the communication with the RyRs, as previous work has shown that RyR regularity and distribution do not change during heart failure. Only LTCCs localized in the crest had abnormally high \( P_o \) which contributed to the pathophysiology of HF suggesting that nanoscale changes in the location of proteins can be detrimental to their function. In fact, it has been proposed that the long open states of the LTCCs are particularly proarrhythmic in the setting of AP prolongation, suggesting that the channels that we found in the crest of failing cells can be a source of arrhythmias.

A number of studies in animal models of HF have demonstrated a reduction in whole-cell Ca\(^{2+}\) current and in the average LTCC density, which appears to be a consequence of the profound loss of TTs, but other studies did not find changes in whole-cell Ca\(^{2+}\). Interestingly, Bryant et al. showed a decreased \( I_{Ca} \) density in the t-tubules and increased \( I_{Ca} \) density on the cell surface in rat HF ventricular myocytes which supports our findings at the single channel level. In human failing cardiac myocytes no significant changes in LTCC density have been observed, however an impaired Cav1.2 trafficking to the TT cell membrane has been suggested. Taking into account the increased \( P_o \) of LTCC observed in our study (Fig.3) and in previous reports, one would expect a reduction of the number of functional channels, although we can’t exclude that a more complex mechanism could be involved. Impaired communication between LTCCs and RyRs, together with an increase in LTCC \( P_o \), may slow down the inactivation time, as demonstrated experimentally.

It remains an open question what is the precise location of functional LTCC on the crests of sarcolemma. Are they sparsely localized on the cell surface or do they organize in special membrane microdomains forming complexes with others proteins? It is plausible that these channels are located in caveolae domains on the plasma membrane of the crest, as we recently demonstrated for extratubular LTCCs in atrial myocytes. Through the function of caveolae-based LTCCs is unknown, Makarewich et al. recently demonstrated that Ca\(^{2+}\) influx through LTCCs within caveolae signaling domains can activate pathological cardiac “hypertrophic” signaling, and this Ca\(^{2+}\) influx can be selectively blocked without reducing cardiac contractility. Whether these channels are associated with the hyperphosphorylated LTCCs observed in our study and the extent to which they may contribute to EADs remain an open question.

Microdomain dependent changes in CaMKII signaling.

For the first time our study reports microdomain-dependent changes in CaMKII-mediated Ca\(^{2+}\) signaling in HF (Fig.3 and 4). CaMKII, a well-known modulator of LTCCs, is upregulated under pathological conditions, resulting in increased LTCC \( P_o \). We extended these finding to show that CaMKII dependent phosphorylation of LTCCs is increased specifically in crest microdomains (Fig.3 and 4) without affecting TT domains. CaMKII also contributed to the occurrence of abnormal calcium
oscillations and lethal arrhythmias (Fig. 4, 6, and 7). CaMKII is a therapeutic target, and CaMKII inhibition provides cardioprotection26, 47. Using experiments and simulations, we elucidated an additional mechanism for the success of CaMKII inhibition in ameliorating HF, namely the inhibition of phosphorylation of dislocated LTCCs48. It has been shown that in HF the $I_{\text{Ca,L}}$ current peak density is not changed, whereas LTCCs density is decreased, suggesting an increase in the activity of the channels42. We propose that the dislocated LTCCs found on the crest can be modulated by CaMKII which could represent a new mechanism explaining this discrepancy.

CaMKII mediated phosphorylation is an essential signaling event in triggering Ca$^{2+}$/CaM-dependent LTCC facilitation which requires the presence of LTCC $\beta$-subunits which can also be directly phosphorylated by CaMKII49. $\beta$-subunits are up-regulated in human failing myocardium and their overexpression correlates with an increase in the $P_o$ of LTCCs50. Thus, we suggest that in failing myocytes, where the loss of TT structure is associated with dislocation of LTCCs to the crests, increase in CaMKII activity would phosphorylate and trigger facilitation of the dislocated LTCCs via two factors: the weak CDI in this microdomain and direct phosphorylation of LTCC $\beta$-subunits, leading to an abnormal LTCC activity.

**Linking sub-cellular changes to arrhythmia propensity in HF.**

Using a new modelling approach spanning from stochastic LTCC gating to arrhythmogenesis at the organ level, we were able to understand how the subcellular changes are able to influence development of arrhythmias. We found that the increase in LTCC $P_o$ though CaMKII hyperactivity is specific to the crest microdomain (Fig. 2), and this produces an increase of the slow inactivation of the $I_{\text{Ca,L}}$ in failing cells (Fig. 5B), as described previously in a stochastic model51. Recently, Morotti et al. using a mathematical model of rabbit action potential linked a decreased CDI to an increase of the slow inactivation of the $I_{\text{Ca,L}}$ and the occurrence of EADs52, which was also observed in the present study.

We demonstrated that in the failing ventricle, microdomain, cell and tissue level abnormalities act in synergy to produce whole organ arrhythmia. It is important to note that HF is a systemic disorder in which affects all of these hierarchical biological levels. For accurate representation of a human HF remodeling process, the membrane model of HF presented in this work used the descriptions provided in Elshrif et al.53 to define the rest of HF ion channel remodeling, outside of $I_{\text{Ca,L}}$. Our study confirms that CaMKII is an important node in this network of changes in the link between HF and arrhythmias. It is involved in disease pathways though the phosphorylation of multiple key proteins, modulating ion channel functioning and effecting gene transcription, metabolism and cell survival54, 55. It has also been linked to the HF-associated up-regulation of the late sodium current, an important contributor to EAD development56, 57. Although many ionic currents can contribute to EAD formation in the settings of HF-associated remodeling, $I_{\text{Ca,L}}$ late appearance plays a central role in providing a regenerative inward current required for EADs to propagate58, 59, thereby causing triggered activity in multicellular tissue60. It has been recently shown that reducing the amplitude of the noninactivating pedestal component of $I_{\text{Ca,L}}$ (i.e. “late” or window $I_{\text{Ca,L}}$) effectively suppressed both H$_2$O$_2$- and hypokalemia-induced EADs61. In non-failing cardiac myocytes, in the settings of potassium current blockade, $I_{\text{Ca,L}}$ was shown to be the main contributor to EADs formation62. Conversely in HF, enhanced late sodium current may also have an important role57, 63. It thus may indicate their additive role in the enhancement of these net inward currents during the plateau phase of the action potential, contributing to EAD development.

**Clinical implications.**

Conventional calcium channel blockers are generally felt to be contra-indicated in HF due to their negatively inotropic effects via inhibiting both peak and window $I_{\text{Ca,L}}$. However such adverse effects may relate to non-specific targeting of LTCCs. The findings from this study can therefore facilitate the development of targeted and effective molecular therapies for preventing sudden cardiac death, without
harmful side-effects, and to steer the development of new and improved approaches to arrhythmia risk stratification of HF patients. In the future, our assay could be used as a development platform for improved therapeutic approaches in combating HF based on the subcellular distribution of their targets. For example, selective block of the non-native LTCC pathway—directly or through subsequent regulatory proteins, including CaMKII—by novel reagents might provide an effective strategy for predicting and ameliorating the risk of sudden cardiac death in patients with cardiac disease.

Limitation.

Failing cardiac myocytes were isolated from human tissue provided with the support of NIHR Cardiovascular BRU at Royal Brompton & Harefield from explanted failing hearts only. Control cardiac myocytes were isolated from human tissue provided with the support of Hammersmith Hospital from patients who underwent mitral valve replacement surgery, with normal ejection fraction values. This indeed may introduce a difference between samples due to the regional heterogeneity within the ventricles, however the differences in human cells between control and failing cases are consistent with the results on rat cells, which are isolated from the same region of the posterior lateral left ventricle free wall. Control human tissue for protein studies was obtained from the left ventricle free wall with the support of University of Sydney.

ACKNOWLEDGMENTS
We thank Dr. Steven Houser for helpful discussion and critical comments on the manuscript. We thank Peter O’Gara for cardiac myocyte isolation and Karina Zimmermann for Western blots. We are grateful to Prof. Cristobal dos Remedios (University Sydney) and Prof. Steve Marston for human heart muscle samples. Human HF tissue provided with the support of NIHR Cardiovascular BRU at Royal Brompton & Harefield.

SOURCES OF FUNDING
This work was supported by Wellcome Trust (JG-WT090594; MBS-WT092852), British Heart Foundation (JG-12/18/30088, ARL-FS/11/67/28954), MRC grant (JG), Imperial College London Rector Award (JG), NIH grants (NT-, R01-HL103428, R01-HL105216 and NT-JG-, RO1-HL126802), NIH Director’s Pioneer Award DP1 HL123271 (NT).

DISCLOSURES
None.
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FIGURE LEGENDS

Figure 1. TT loss in ventricular myocytes from failing human and rat hearts. (A) Example confocal images of human control and failing (top) and rat control and failing (bottom) cardiac myocytes showing membranes stained with di-8-ANNEPS, scale bar 10µm. (B) TT density in control and failing cells (human control n=20, failing n=8; rat control n=20, failing n=20; **P<0.01 by unpaired Student t-test). (C) Example SICM scans from a 10µm x 10µm portion of cell membrane shows regular undulations, indicating spatially alternating TT invaginations and surface membrane “Crests” in human (top left) and rat (bottom left) control cardiac myocytes, that are relatively absent in failing cells (right). (D) Z-groove index in human and rat failing cells normalized to control average value (human control n=30, failing n=59; top, P<0.01; rat control n=91, failing n= 122; bottom, P<0.001 by unpaired Student t-test).

Figure 2. Abnormal LTCC localization and function in failing cardiac myocytes. Chance of obtaining a LTCC current (% occurrence) in human (A) and rat (B) control and failing cells (*** denotes P<0.01 by Fisher’s exact test). P_e during a maximum activating voltage step (to -6.7mV) in human (C) and rat (D) failing cells (human control, TT n=6, crest n=1; failing, TT n=6, crest n=9, *denotes P<0.05, **denotes P<0.01 by Mann-Whitney test) (rat control, TT n=19, crest n=4; failing, TT n=11, crest n=13, ***denotes P<0.001 by Mann-Whitney test ). Representative single channel traces at -6.7 mV showing LTCC activity in human (E) and rat (F) cardiac myocytes.

Figure 3. LTCCs at crests in rat failing myocytes are hyperphosphorylated by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). (A) Mean phosphorylated CaMKII (Thr286) from whole cell lysate in control and failing cardiac myocytes. A representative blot is shown above the graph. Results are normalized to α-tubulin (B) Mean p-CaMKII from whole tissue from control and failing human samples. A representative blot is shown above the graph. Results are normalized to GAPDH. (C) Mean p-CaMKII from whole cell lysate in failing cardiac myocytes with or without CaMKII inhibition. A representative blot is shown above the graph. Results are normalized to α-tubulin. (D) LTCC P_e in control and failing rat myocytes, under control conditions and following CaMKII inhibition with 5 μM KN-93 or with 5 μM AIP (control TT n=19; control TT+KN-93 n=5; failing TT n=11; failing TT+KN-93 n=4; control crest n=4; failing crest n=13; failing crest+KN-93 n=9; failing crest+AIP n=3. * denotes P<0.05, ** denotes P<0.01 by Kruskal-Wallis test).

Figure 4. Ca^{2+} oscillations that develop in failing but not in control rat cardiac myocytes are CaMKII-dependent. (A) Left, Spontaneous [Ca^{2+}]i oscillation occurred at a pacing rate of 0.5Hz during the decay phase of calcium transients in failing rat cardiac myocytes only. Right, percentage of cells exhibiting Ca^{2+} oscillations (control n=106, failing =73, * denotes P<0.05 by Mann-Whitney test). (B) Left, Example of Ca^{2+} transients recorded from the same failing cardiac myocytes before and after treatment with KN-93. Right, Number of cells (in %) exhibiting Ca^{2+} oscillations (n=2 rats, 32-114 cardiac myocytes in each group; *denotes P<0.05 for failing vs. control; ^P<0.05 for KN93 effect within the same group by Mann-Whitney test). Saline mimics no treatment.

Figure 5. Simulations demonstrated increased Po of LTCCs at failing Crest sites, as in experiments, and slowed inactivation. (A) Comparison between simulated and experimental P_e values for 1s voltage steps to -6.7 mV (average of 500 stochastic sweeps in simulations). (B) Simulated whole cell TT and crest LTCC current (I_{Ca,L}) in control (black), and failing (red) cells. Traces in right column were normalized to allow visual comparison of decay rates.

Figure 6. Simulations demonstrating the development of pro-arrhythmic triggers (early afterdepolarizations, EADs) in failing human ventricular (endocardial) myocytes. Rows from top to bottom show simulated action potentials, TT (black) and Crest (red) I_{Ca,L}, TT and Crest CaMKII activity (0.0 – none, no LTCCs phosphorylated; 1.0 – maximal, all LTCCs phosphorylated), and TT and crest Ca^{2+}
concentrations (i.e. concentrations in the dyadic and sub-sarcolemmal volumes). Columns from left to right show cases of control, failing, and failing cells with CaMKII block.

**Figure 7.** Whole heart simulations predict the formation of arrhythmogenic triggers and reentrant arrhythmia in human heart failure. 1Hz pacing with a single skipped beat was from the apex. (A) Action potential traces at endocardial sites “i-iii” for control and failing hearts. Small dashed gray lines relate stimuli (solid gray tick marks) to resulting action potentials at the sites. In the failing heart, EAD triggers (red, arrows) appeared near site “ii” and propagated outwardly to other sites (gray lines emanating from EADs and triggered action potentials). Vertical semi-transparent bands denote time windows over which activation and voltage maps are shown in remaining panels. (B1 and B2) Activation maps, showing the time at which membrane voltage first crossed activation threshold, in the control and failing ventricles during the respective time windows B1 and B2 shown in panel A. (C) Progression of activation recovery during the time window C shown in panel A (from 4.0s to 6.0s, time points separated by 1/3s). An island of EAD generating tissue can be seen near site “iii” in snapshot 1. Reentry developed following the propagation of triggered activity emanating from the EAD site (path summarized in the cartoon at bottom right; arrows show general direction of propagation and should not be taken as belonging to specific wavefronts).
Novelty and Significance Section

What Is Known?

- Heart failure (HF) alters cardiac function as well as the functionality of ion channels.
- Cells from HF models show a progressive loss of membrane structures and organization.
- L-type calcium channels (LTCC) are considered an important component of alterations seen in HF cells.

What New Information Does This Article Contribute?

- Loss of structure in HF cardiac myocytes leads to redistribution of LTCCs.
- Relocated LTCCs have different characteristics and may be a source of arrhythmias.
- Translational modelling studies suggest that these specific LTCCs could be a potential drug target.

HF is a complex and multifactorial disease. At the single cell level, HF causes hypertrophy, disorganization of the T-tubular structures, and alterations of ion channels profiles, including the LTCCs. To assess the impact of these alterations on ion channels, we studied isolated myocytes from the failing human heart. We found that the loss of T-tubules leads to the redistribution of a portion of LTCCs on the cell surface. This redistribution leads to a dramatic increase in the activity of the LTCCs. They become a possible source of arrhythmias (due to the manifestation of early after depolarizations) and are partly controlled by the increase in the CaMKII activity seen in HF. We extrapolated these findings observed at the single cell level to whole heart using mathematical modeling, to deduce the consequence of the redistribution of LTCCs on the propagation of arrhythmias. Our results suggest that non-physiological ion channels in specific microdomains could be novel therapeutic targets in HF.
FIGURE 1

A

Confocal (di-8-Annels) (di-

Human Control Human Failing

Rat Control Rat Failing

B

Human

TT density(%) Control Failing

Rat

TT density(%) Control Failing

C

Human Control Human Failing

Rat Control Rat Failing

D

Human

Normalize Z-groove index Control Failing

Rat

Normalize Z-groove index Control Failing
FIGURE 4

A

Arrhythmogenic \( [\text{Ca}^{2+}]_i \) oscillations

Control

Failing

\( [\text{Ca}^{2+}]_i \)

500 ms

% of cells exhibited oscillations

Control

Failing

B

No treatment

\( \text{Ca}^{2+}_i \)

KN-93 (5μM)

\( \text{Ca}^{2+}_i \)

1s

% of cells exhibited oscillations

No treatment

Saline

KN-93 (5μM)
FIGURE 5

A

Po at -6.7 mV (%)

|        | TT  | Crest |
|--------|-----|-------|
| nonfailing |     |       |
| exp.    | 4   | 12    |
| sim.    | 4   | 12    |
| exp.    | 4   | 12    |
| sim.    | 4   | 12    |

|        | TT  | Crest |
|--------|-----|-------|
| failing |     |       |
| exp.    | 16  | 12    |
| sim.    | 16  | 12    |

B

simulated whole cell $I_{\text{CaL}}$

nonfailing

TT $I_{\text{CaL}}$

Crest $I_{\text{CaL}}$

-6.7 mV

failing

normalized

100 ms
FIGURE 7

A

B1 nonfailing failing

B2

t=1.0s 1.6s

t=4.0s 4.3s

C failing voltage maps

t=4.0s

5. 6. 7.

t=6.0s
Microdomain-Specific Modulation of L-type Calcium Channels Leads to Triggered Ventricular Arrhythmia in Heart Failure

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Circ Res. published online August 29, 2016;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2016/08/28/CIRCRESAHA.116.308698
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SUPPLEMENTAL MATERIAL

Microdomain-Specific Modulation of L-type Calcium Channels Leads to Triggered Ventricular Arrhythmia in Heart Failure

Jose L. Sanchez-Alonso, Anamika Bhargava, Thomas O'Hara, Alexey V. Glukhov, Sophie Schobesberger, Navneet Bhogal, Markus B. Sikkel, Catherine Mansfield, Yuri E. Korchev, Prakash P. Punjabi, Viacheslav O. Nikolaev, Natalia A. Trayanova, Julia Gorelik

1. Heart failure rat model and rat cardiomyocyte isolation

Adult male Sprague-Dawley rats (250-300g) underwent proximal coronary ligation to induce chronic myocardial infarction as described before.1,2 Briefly, rats were anesthetized with 2% isoflurane, intubated, and ventilated after preoperative buprenorphine (0.03 mg/kg SC) injection. The thorax was shaved and sterilized with 2% w/v chlorhexidine gluconate in 70% v/v isopropyl alcohol. A left thoracotomy was performed, and the left anterior descending coronary artery was ligated with 6-0 silk. Sham ligation was used as control. Sixteen weeks later, in vivo PV analysis was performed using the 2-F Millar microconductance catheter (SPR838; Millar Instruments) via an apical approach under isoflurane (1.5%) anesthesia. Steady-state data (left ventricle end systolic and diastolic dimensions, and left ventricle ejection fraction) were recorded after 15 minutes’ stabilization. Data were recorded using CHART 5.5 software (AD Instruments) and analyzed off line using PVAN 3.6 software (Millar Instruments). Hearts were explanted, weighed, and prepared for cell isolation.

This heart failure model recapitulates many features of chronic heart failure in patients including adverse remodeling of the organ, characterized by left ventricle and left atria dilatation, reduced ejection fraction, raised filling pressures and elevated serum natriuretic peptides.2-4 In particular, heart failure rats exhibited left ventricle dilatation, reduced ejection fraction and increase in the cell length (Online Figure I). Ventricular cells from this model have been studied extensively and are well characterized at structural, biochemical, molecular, Ca\(^{2+}\) handling, and electrophysiological levels.2,4,5
Ventricular cardiomyocyte isolation was done as previously described. Briefly, Sprague-Dawley rats (150–250 g) were anesthetized with 5% isoflurane-95% O₂ and then killed by cervical dislocation. Hearts were fast extracted and placed in Tyrode solution containing in (mmol/L): 140 NaCl, 6 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose and 10 HEPES, adjusted to pH 7.4 with 2 mmol/L NaOH. Using aortic cannulation with the Langendorff setting, the hearts were perfused with Tyrode solution for 5 min, then with low Ca²⁺ solution containing in (mmol/L): 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES, 5 nitrilotriacetic acid, and 0.04 CaCl₂, adjusted to pH 6.96 with 2 mmol/L NaOH for 5 min, and finally for 10 min with enzyme solution containing in (mmol/L): 120 NaCl, 5.4 KCl, 5 MgSO₄, 5 sodium pyruvate, 20 glucose, 20 taurine, 10 HEPES, and 0.2 CaCl₂, pH 7.4 with collagenase (1 mg/ml; Worthington) and hyaluronidase (0.6 mg/ml; Sigma-Aldrich).

Cardiomyocytes were plated on dishes coated with laminin and left to stick to bottom for at least 45 minutes before experiments. Cardiomyocytes were used on the same day as isolation.

2. Patients groups and human cardiomyocyte isolation

To control for factors of regional-dependent heterogeneity, the presence of acute ischemia, and ischemic injury, the current study was conducted on dilated end-stage cardiomyopathic human hearts (n=6, patients, average age 48±5 years, two females and fourth males, Online Table I) with the approval from Bromton Harefield & NHLI Research Ethic Committee (Ref 01-194). For comparison, we used ventricular left-over specimens obtained with the consent from non-failing patients (n=5, average age 69±2 years, two females and three males, Online Table II) during valve replacement procedure at Hammersmith Hospital, Imperial College London, London, UK. Although non-failing control patients were complicated by various factors, including age, early stage hypertrophy, atrial fibrillation, and coronary disease, they possessed normal left ventricle function (ejection fraction >60%).

Failing ventricular cardiomyocytes were isolated from the apical section of the posterior-lateral left ventricle free wall by enzymatic digestion as previously described. Briefly, individual specimens were transferred to ice-cold calcium free Krebs-Ringer saline solution consisting of (in g/L): 7.012 NaCl, 0.402 KCl, 1.332 MgSO₄, 0.55 Pyruvate, 3.603 Glucose, 2.502 Taurine, 2.383 HEPES, 1.286 Nitrilotriacetic Acid; pH = 6.96. Connective and adipose tissue were removed and approximately 500 mg of myocardial tissue was minced.
with razor blades in small cubes (approx. 1-2 mm³). Then, the tissue pieces were washed with fresh Ca²⁺-free Krebs-Ringer solution 3 times for 3 min each at 37°C. After wash, cardiac tissue was incubated for 25 min in 10 ml of Krebs-Ringer solution containing (in g/L): NaCl 7.012, KCl 0.402, MgSO₄ 1.332, Pyruvate 0.55, Glucose 3.603, Taurine 2.502, HEPES 2.383; pH = 7.4, supplemented with 200 nM CaCl₂ and Proteinase type XXIV (0.36mg/ml; Sigma-Aldrich) under gentle agitation. The partially digested tissue was transferred to 10 ml of Krebs-Ringer saline supplemented with collagenase type XIV (1mg/ml Sigma-Aldrich). The tissue was incubated thrice with this solution for 10 min each at 37°C with gentle agitation. Usually, cardiomyocytes were visible by phase contrast light microscopy after the first incubation step, with the biggest amount of cells after the second incubation step. After each incubation step, the supernatants were transferred to a tube and centrifuged at 600 rpm for 3 min. The pellets were re-suspended in 2-3 mL of Krebs-Ringer solution. After isolation, human cardiomyocytes were plated following the same protocol as rat cardiomyocytes. Non-failing control human ventricular cardiomyocytes were isolated from the left ventricle papillary muscles.

3. T-tubule labelling and analysis

Di-8-ANEPPS was excited at a wavelength of 488 nm and confocal images were taken at 63x magnification using a Zeiss LSM-780 inverted confocal microscope. The 40x5 microns size area inside the sarcolemma that did not coincide with the cell nuclei was chosen, automatically thresholded into binarised, and the TT density was determined as ratio of black pixels versus white pixels. The TT regularity was calculated through a single dimension Fourier transformation using a custom-written macro for Matlab (The MathWorks, Inc., Natick, MA, USA) as described before.⁶

For the visualization of the TT network structure in combination with SICM, cardiomyocytes were stained with the fluorescent dye Di-8-ANEPPS (10µM) for 1 min. Di-8-ANEPPS was excited at a wavelength of 473 nm with a StradusTM473 laser (Vortran) and confocal images were taken at 100x magnification using a Photomultiplier Detection System (PTI).

4. Super-resolution scanning patch-clamp with pipette clipping modification

This technique combines scanning ion conductance microscopy (SICM) and patch-clamp electrophysiology with a pipette clipping modification to increase the throughput of recording
ion channel activity from cell surface microdomains. SICM uses a sharp pipette (~100 nm inner diameter, ID, 100 MΩ resistance) as a scanning probe to generate high resolution topography images of live cells.\(^7\) SICM is based on the principle that the ion current through the electrolyte-filled micropipette is partially occluded when the pipette approaches the surface of a cell.\(^8\) Therefore, the position of the tip of the pipette relative to the cell surface strongly influences the ion current through the pipette. This ion current is digitized and fed into the feedback and scan control system which provides the feedback signal to control the vertical position of the pipette keeping the pipette-sample separation constant. The pipette raster scans the sample and generates a 3D topography image of the cell surface.

Next, to clip the pipette tip to generate a wider pipette necessary to increase the chances of getting an ion channel, the pipette is positioned above a free surface away from cells. Then, moving down with high velocity the pipette is allowed to impact onto the surface and the tip of the pipette clips in a controlled manner.\(^7\) This is possible because at a high enough velocity of approaching the surface the feedback control of the scanning system cannot generate enough upward movement of the piezo-drive quickly enough to avoid contact with the surface. With this method at 500 nm/ms the pipette tip is clipped to approximately ~350 nm ID, 30 MΩ resistance. The pipette is then positioned back onto the cellular microdomain of choice (in this case T-tubule or crest) taking help of the coordinates from the image acquired before clipping. The feedback is turned off; pipette is lowered until it touches the cell surface and a very gentle suction is applied to form a gigaseal (Online Figure II). Cell-attached single channel recordings are then performed by the conventional patch-clamp technique.

5. Electrophysiological recordings

5.a. Cell attached LTCC

Cell-attached patch-clamp recordings of single LTCC currents were performed at room temperature using the following solutions; external solution containing in (mmol/L): 120 K-glucuronate, 25 KCl, 2 MgCl₂, 1 CaCl₂, 2 EGTA, 10 Glucose, 10 HEPES, pH 7.4 with NaOH, ~290 mOsm; internal recording solution containing in (mmol/L): 90 BaCl₂, 10 HEPES, 10 Sucrose, pH 7.4 with TEA-OH, ~250 mOsm. The pipette used for cell attached recordings had a typical resistance of approximately 30 MΩ. Currents were recorded using Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA), controlled and monitored using pClamp software version 10 (Axon Instruments). Single LTCCs were identified and
characterized by their voltage dependent properties. To generate a current-voltage (I–V) relationship, the membrane under the patch was held at a voltage of -80 mV and voltage pulses were applied from -30 to +30 mV in the incremental steps of 10 mV. Analysis was performed as previously described. Single channels were sampled at 10 kHz and filtered at 2 kHz (-3 dB, 8-pole Bessel). Single channel data were analysed using Clampfit version 10.2. A liquid junction potential, calculated to be -16.7 mV, was corrected from the data shown.

Every time a channel is recorded a minimum of 100 sweeps at -6.7 mV (after liquid junction potential correction) and 3 full protocols at different voltage steps were done. All the sweeps are checked for the presence of more than one channel. When no second level of spikes is observed in any of the sweeps the recording is classified as one channel. Overall, from 89 recordings showing activity in this work, 70 (79%) of them were classified as one channel, 13 (14%) show two channels, 5 (6%) triple channels, and only in one occasion (1%) the recording showed 4 channels, and this is similar between TT and Crest regions. From 22 recordings of human cells 64% were classified as one channel versus rat cells where from 67 recordings 84% were classified as one channel. Seal data is showed in detail on Online Table III.

Occurrence of LTCCs was calculated as the percentage of recording showing activity versus the total number of recordings, higher occurrence can be interpreted as a higher density of channels on that specific location or group.

The open probability (Po) was averaged from 10-20 sweeps at -6.7 mV for each cell. Each cell was recorded only once, and only one value of Po per cell was used. The total number of channels in the recording was input into pClamp software to calculate the Po and the peak current (calculated as the overall average current) of one single channel.

5.b. Action potential recordings

APs were recorded from isolated LV rat cardiomyocytes using the current-clamp configuration of the patch-clamp technique (Figure S12) with the external recording solution containing (mmol/L): 120 NaCl, 5.4 KCl, 0.2 CaCl₂, 5 MgSO₄, 10 HEPES, 20 glucose, 5 pyruvate sodium, 20 Taurine, pH 7.4 with NaOH, ~300 mOsm, and the internal pipette solution containing (mmol/L): 120 KCl, 5 EGTA, 10 HEPES, 0.5 MgCl₂, 1 CaCl₂, 5 Mg-ATP, pH 7.3 with KOH, ~290 mOsm. Currents were recorded using Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA), controlled and monitored using pClamp software version 10 (Axon Instruments). Patch pipettes had typical resistances of 8-10 MΩ.
The bath was connected to the ground via an Ag–AgCl pellet. Data were sampled at 10 kHz. All recordings were performed at 33-35°C. APs were elicited with 5-ms current pulses at 1Hz. AP characteristics were analysed using pClamp software.

5.c. Whole-cell I_{CaL}

Macroscopic Ca2+ currents were recorded using the whole-cell patch-clamp technique (Figure S13) with the external recording solution containing (mmol/L): 1 CaCl₂, 0.5 MgCl₂, 5 HEPES, 140 choline chloride, 5 CsCl, 5.5 glucose, pH 7.4 with CsOH, ~305 mOsm, and the internal pipette solution containing (mmol/L): 130 Cs-methanesulphonate, 11 EGTA, 10 HEPES, 2 MgCl₂, 5 Mg-ATP, 0.3 Na-GTP, pH 7.2 with CsOH, ~290 mOsm. Currents were recorded using Multiclamp 700B amplifier (Axon Instruments, Foster City, CA, USA), controlled and monitored using pClamp software version 10 (Axon Instruments). Patch pipettes had typical resistances of 1–2 MΩ. The bath was connected to the ground via an Ag–AgCl pellet. Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier and sampled at 10 kHz. The amplifier was also used for capacitive transient and series resistance compensation between 60 and 70% on each cell. Leak subtraction of leakage currents was performed with Clampfit during off-line analysis. All recordings were performed at 33-35°C. I–V relationships were obtained by holding the cells at a potential of −50 mV before applying 100 ms pulses to potentials from −70 to +40 mV every 5s in 10 mV increments. I–V relationships were fitted with the modified Boltzmann equation, \( I = \frac{G_{\text{max}} \times (V_m - E_{\text{rev}})}{1 + \exp\left(\frac{V_m - V_{0.5a}}{K_a}\right)} \), where \( V_m \) is the test potential, \( V_{0.5a} \) is the half-activation potential, \( E_{\text{rev}} \) is the extrapolated reversal potential, \( G_{\text{max}} \) is the maximum slope conductance and \( K_a \) reflects the slope of the activation curve.

6. Single Channel Human L-type Ca^{2+} Current (I_{Ca,L})

6.a. Markov Formulation

Human ventricular cell electrophysiological behavior was represented by the O'Hara-Rudy model, selected because L-type Ca\(^{2+}\) current, \( I_{\text{Ca,L}} \), in the model was based on undiseased human data, and because accurate representations of heart failure ion channel remodeling in transmural cell types of the model are available. The O’Hara-Rudy \( I_{\text{Ca,L}} \)
follows the Hodgkin-Huxley (HH) formalism. However, we needed to model stochastic single channel behavior so that we can determine the channel open probability ($P_o$) for comparison with human experimental data. We thus converted the HH $I_{Ca,L}$ model to its Markov-equivalent representation.

**Online Figure IV** shows the Markov equivalent of the O'Hara-Rudy $I_{Ca,L}$. There are 32 states in each of the CaMKII un-phosphorylated and CaMKII phosphorylated modes (64 total states). In each mode, there are 6 open states. Nested cubes in each mode are identical, except for open states, and include activation/deactivation by the “d” gate (right/left) and inactivation/recovery by the “f” (up/down) and “fca” (in/out of page) gates. The inner- and outermost pairs of nested cubes are connected by the slow recovery “jca” gate. The gate “nca” is Ca$^{2+}$-sensitive and specifies whether inactivation is voltage (“f”), or Ca$^{2+}$ (“fca”) driven. The innermost cube connects with the third cube, and the second cube connects with the outermost one via the “nca” gating. Thus, the four discrete combinations of “jca” and “nca” define the four nested cube (from innermost to outermost cube: jca=1, nca=0; jca=0, nca=0; jca=1, nca=1; jca=0, nca=1).

Both “f” and “fca” gates have fast and slow gating modes. CaMKII un-phosphorylated channels inactivate in both fast and slow gating modes. Inactivation occurs via the slow mode only for channels phosphorylated by CaMKII.

6. **b. Stochastic Sweeps using Gillespie Exact Algorithm**

To evolve the channel gating in response to a 1-s voltage change step to -6.7 mV (from resting state, marked *), we used the Gillespie Exact Algorithm, implemented in Matlab (The Mathworks Inc.). The built-in pseudo-random number generator function, “rand”, was seeded to the system clock and used twice at each state change step, as required by the algorithm. Example single channel sweeps are shown in **Online Figure VIII**.

6. **c. Reverting to Hodgkin-Huxley Formalism**

Once single channel current simulation results were generated and validated with experimental $P_o$ measurements, the $I_{Ca,L}$ model was reverted back to an HH formulation. The HH formulation retained the CaMKII mode definitions and the behavior of the equivalent Markov version, but could be numerically integrated rapidly and with ease in cell (the O'Hara-Rudy model) and tissue simulations. Failing Crest LTCCs were assumed to operate in CaMKII phosphorylated mode, based on experimental findings. Their inactivation was strictly via the slow gating mode. Failing LTCCs in TTs and control LTCCs were sensitive to
standard CaMKII levels in the cell, and so inactivation was both fast and slow. Ensemble current computed by summation of single channel sweeps matched the deterministic HH current, as expected (Online Figure V).

7. Models of Human Control Nonfailing and Failing Ventricular Myocytes

7. a. Fast Na⁺ Current ($I_{Na}$)

As noted online in the PLoS Computational Biology comments associated with the O'Hara et al. 2011 publication of the O'Hara-Rudy ionic model,9 we replaced the original $I_{Na}$ with the formulation used in the ten-Tusscher et al human ventricular cell model.10 The ten-Tusscher et al formulation was based on the same set of human experimental data. The ten-Tusscher et al myocyte model and its $I_{Na}$ have been used extensively to simulate action potential propagation in tissue (e.g. 11, 12).

7. b. Sub-sarcolemmal Volume

In the Grandi et al human cell model,13 SL volume was 0.0308 times that of the bulk myoplasm and 3.7106 times that of the dyad (or junctional) volume. However, bulk to dyad volume ratio is different in the Grandi et al and O’Hara-Rudy models. The Grandi et al model definition could be directly adopted by the O’Hara-Rudy model as either the ratio of bulk myoplasm volume to SL volume, or as dyadic volume to SL volume. In this study, SL volume in the O’Hara-Rudy model was set to be the average of these two options (0.04755 times the whole cell volume).

As was done by Grandi et al and Shannon et al, we set the flux rate between SL and myoplasm to be 0.2213 times that between dyad and SL. We assumed that the geometry of the dyad/SL interface was identical to the original O’Hara-Rudy dyad/myoplasm interface.

Ca²⁺-dependent inactivation (CDI) of $I_{Ca,L}$ occurs regardless of Ca²⁺ origin, whether from Ca²⁺ release from RyRs in the dyadic volume or from the channel’s own flux. The original O’Hara-Rudy myocyte model did not include a sub-sarcolemmal (SL) volume and thus did not allow for Ca²⁺ accumulation near the intracellular mouth of Crest LTCCs. In the human ventricular cell model by Grandi et al.,13 an SL volume was included (originally incorporated in the Grandi et al model predecessor, a rabbit ionic model by Shannon et al 14). We incorporated SL volume and related fluxes in the O’Hara-Rudy myocyte model based on the work of Grandi et al and Shannon et al.13, 14
As was done by Grandi et al and Shannon et al, we set the flux rate between SL and myoplasm to be 0.2213 times that between dyad and SL. We assumed that the geometry of the dyad/SL interface was identical to the original O’Hara-Rudy dyad/myoplasm interface.

7. c. LTCC Current Density at TT and Crest Locations

The original O'Hara-Rudy model included LTCCs exclusively at TT sites with dyadic intracellular face. In the present simulations, LTCCs in the TTs sensed and contributed to dyadic Ca\(^{2+}\); channels newly added to the Crest sensed and contributed to SL Ca\(^{2+}\). Channel kinetics and all equations governing behavior were identical in TT and crest. That is, changes in current function were solely based on extrinsic environmental changes, not intrinsic changes in kinetics.

In each of TT and crest locations, LTCC permeability, PCa, representing whole-cell current density in TTs (PCa\(_{TT}\)) and crest (PCa\(_{Crest}\)) needed to be defined. We utilized experimental data obtained in this study to assign values to PCa\(_{TT}\) and PCa\(_{Crest}\) in control and failing human cells. The data used were: % occurrence of LTCCs, LTCC single channel current amplitude, and in failing cells, the degree of TT loss. Thus, we arrived at the following values for fractional PCa (fPCa) in control cells: fPCa\(_{control,TT}\) = 0.75 and fPCa\(_{control,Crest}\) = 0.25 (see manuscript Figure 2A). The original O'Hara-Rudy model PCa value was multiplied by these fractions to get accurate current density at TTs and crest in control myocytes.

Values for fractional PCa (fPCa) in control cells were: fPCa\(_{control,TT}\) = 0.75 and fPCa\(_{control,Crest}\) = 0.25 (see manuscript Figure 2A). The original O'Hara-Rudy model PCa value was multiplied by these fractions to get accurate current density at TTs and crest in control myocytes.

In failing human cells, our experimental findings (data not shown) indicated that LTCC single channel amplitude was slightly, but statistically significantly greater in TTs (1.2 ± 0.07 -fold greater) and slightly but statistically significantly lower at crest sites (0.85 ± 0.06 times) when compared to control TT amplitude, the latter being our reference amplitude. Thus, the LTCC % occurrence (as a fraction of total LTCC occurrence), (manuscript Figure 2A), was multiplied by these single channel amplitude factors (1.2 and 0.85 for TT and Crest, respectively). For failing Crest, this was fPCa\(_{failing,Crest}\) = 0.75*0.85 = 0.63.

To arrive at a value for fPCa\(_{failing,TT}\), an additional factor accounting for the degree of TT loss was incorporated. This was necessary because TT loss reduces whole cell density of LTCC TT channels, which lowers fPCa\(_{failing,TT}\). Loss of TTs in failing human myocytes was
the average of fractional TT density (failing/control = 0.51 ± 0.08) and fractional Z-groove ratio (failing/control = 0.41 ± 0.06, average of 0.54). Thus, \( fPCa_{failing,TT} = 0.75 \times 1.2 \times 0.54 = 0.486. \)

Finally, experimental evidence from human myocytes has indicated (see for example \(^{15} \)) that HF ion channel remodeling does not have an effect on peak \( I_{CaL} \). Therefore, we adjusted the whole cell LTCC peak current in failing myocytes to match that of control.

In summary:

- \( PCa_{control,TT} = 0.75 \)
- \( fPCa_{control,Crest} = 0.25 \)
- \( PCa_{failing,TT} = 0.3846 \)
- \( fPCa_{failing,Crest} = 0.4985 \)

7. d. \( Na^+/Ca^{2+} \) Exchanger (NCX)

The original O'Hara-Rudy model assumes that 20% of NCX channels reside on the surface membrane. In the present simulations, the percent of NCX residing at crest sites (i.e. non-TT-residing NCX) was increased in accordance with the degree of TT loss, assuming that NCX migrates to the Crest in a way similar to LTCCs migration following TT loss. Since the value for the fraction of intact TTs was 0.54 (see above), 46% (100%-54%) of the original 80% of NCX residing in TTs were relocated to the Crest in failing myocytes. The importance of accounting for NCX re-distribution following TT degradation arises from the fact that it affects concentrations in the SL volume.

7. e. “Orphaned” RyRs

With TT loss, the association between LTCCs and RyRs is also lost, affecting \( Ca^{2+} \) induced \( Ca^{2+} \) release. The “orphaned” RyRs generate poorly coordinated \( Ca^{2+} \) release events.\(^{16} \) This was represented in the HF myocyte model by defining the \( I_{CaL} \) trigger as \( I_{CaL,TT} \) and by restricting RyR flux (“Jrel”) to the portion of remaining TTs (i.e. to 54% of the original number of TTs). This resulted in weakening of the trigger/response coupling between LTCCs and RyRs in failing cells, and in turn, affected LTCCs either directly or indirectly based on \( Ca^{2+} \) in their TT of Crest location, respectively.

A more biophysically accurate and complete representation of RyR orphaning requires high sub-cellular spatial resolution not captured by the common pool dyadic volume in the O'Hara-Rudy model. Such representations are required for simulation of \( Ca^{2+} \) waves,
which can occur in failing myocytes, but are not the focus of this study. The above, more phenomenological representation of RyR orphaning kept the focus of this research on LTCCs in the failing cell, and enabled implementation of the myocyte model in an organ-level electrophysiological model to study the downstream electrophysiological consequences. i.e. arrhythmogenesis.

7. f. Heart Failure Remodeling

A recent publication provided a comprehensive literature review of heart failure remodeling data in the human myocyte and a summary of how the data have been used in previous computational models. The publication used the O’Hara-Rudy model and presented endocardial and epicardial transmural definitions of heart failure ion channel remodeling, implemented as scaling of conductances to best represent the human dataset. We used the descriptions provided in Elshrif et al. (table 2) to define the rest of HF ion channel remodeling (outside of I_{Ca,L}) in our model.

Holzem et al. examined rapid delayed rectifier current (I_{Kr}) remodeling, providing new human data and a human myocyte model based on the Grandi et al framework; simulations of myocyte behavior in HF best matched experimental data when I_{Kr} magnitude was reduced by 50%. We used this value for I_{Kr} magnitude remodeling in the failing myocyte model in the present study.

A ~3-fold increase in CaMKII activity has been reported in human failing versus control cells. In the failing myocyte model we thus assumed that CaMKII at Crest/SL sites was maximal, while in the rest of the failing cell it was multiplied by a factor of 3.

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Online Figure I. Characteristics of the rat model of heart failure. (A) Heart weight to tibia length ratio was significantly increased in heart failure (control n=8 vs. failing n=16, P<0.05). (B) Ejection fraction was significantly decreased in heart failure (LVEF, left ventricular ejection fraction, control n=6 vs failing n=14, P<0.001). (C) Cell size was significantly increased in heart failure (control n=20 vs. failing n=20, P<0.001). (D) Total arrhythmias, (P<0.001) (E) Complex ventricular arrhythmias (P<0.01) (F) VT % cases.
Online Figure II. Super-resolution scanning patch-clamp method. A topographical image of the cardiomyocyte generated by SICM with a 100 nm nanopipette. The inner tip diameter of the nanopipette is widened to ~350 nm by controlled clipping to increase the area of attachment. First, the pipette was navigated to an area free of cardiomyocytes, the fall rate was increased and pipette was allowed to impact on surface. As a result the pipette breaks its tip and increases its diameter because of the conical shape of the pipette. Pipette tip breaking resulted in stepwise increases of the pipette current as its resistance dropped. The breaking was automatically stopped by returning the fall rate to baseline (60 nm/ms) once the pipette current reached a desired level. After clipping, the pipette is lowered to a specific location (TT or crest) and a gigaseal is established. Single ion channels are recorded in cell-attached mode. Insets show the pipette tip size before and after clipping.
Online Figure III. Clipping procedure does not change pipette position. T-tubule location on the surface scan before (left) and after (middle) clipping. After clipping the pipette resistance (Rp) reduced from 120 MΩ to 38 MΩ. Surface deepness thresholding was used to visualize location of T-tubules before (green) and after clipping (red). Because the pipette was held vertically at all times, x, y coordinates of the pipette tip did not change.
Online Figure IV. State diagram of the Markov model equivalent of the O'Hara-Rudy Hodgkin-Huxley (HH)-formalism-based L-type Ca\(^{2+}\) current formulation. Vertices of nested cubes on the left show connected states in the non-CaMKII-phosphorylated model. To the right are CaMKII-phosphorylated model states, distinguished by 2.5-fold slower inactivation ("f\(_{CaMK}\)" and "f\(_{Ca,CaMK}\)" in purple and pink are slower than "f" and "f\(_{Ca}\)" in red and orange, left). When CaMKII phosphorylated, inactivation followed “slow” rather than “fast” paths (indicated by thick “slow” and thin “fast” connections in purple and pink). Colors and directional descriptors below indicate correspondence between the HH-based gates and Markov state transitions, as drawn in the diagram. Open states are marked by black dots.
Online Figure V. Examples of random single channel current sweeps. The Gillespie Exact Monte Carlo Algorithm was used to calculate stochastic gating in response to a 1 s voltage step to -6.7 mV. Sweeps show early and infrequent openings without CaMKII (left column of sweeps) and frequent and late openings with CaMKII phosphorylation.
Online Figure VI.

Online Figure IV. Examples of summation of an ensemble of 500 stochastically gating LTCCs. Ensemble traces in red were compared with the deterministic HH-based current (blue), illustrating that the single channel model produces the expected results in response to a voltage step to -6.7 mV.
Online Figure VII. Human heart control and failing computational models. (A) MRI-based geometry of the human ventricular model. (B) Transmural cell types. (C) Transmural tissue conductivity values incorporated in the model, based on experimental data regarding transmural Cx43 distribution from human left ventricular wedges, as measured in Glukhov et al.\textsuperscript{19} 2010. The values vary across the septum and free wall following the gradient pattern shown in panel B.
Online Figure VIII. TT openings decrease in failing cells. (A) Confocal and SICM images of the same 17.5 µm x 17.5 µm patch of membrane. The images demonstrate correlation between the two measurement modalities in both number and localization of TTs. In confocal images, di-8-ANNEPS staining (10 µM) marks the membrane. SICM images the membrane surface topology. (B) Left, SICM scans of a control and failing cardiomyocyte, black holes on the surface indicate TT openings. Right, Quantification of TT openings (control n=41 vs. failing n=44, P<0.001). (C) Top, power spectrum retrieved from a 2D Fourier transformation of confocal images of TT from a control and failing cardiomyocyte. Bottom, quantification of peak power values (in arbitrary units) at the dominant frequency (control n=11 vs. failing n=11, P<0.001).
Online Figure IX. Increased LTCC activity in the crest of failing cells is not due to changes in current amplitude. (A) Voltage dependence of single LTCC currents in the TTs and in the crest of control and failing cardiomyocytes. (B) Representative single channel traces at indicated voltages. Downward deflections are opening of single LTCC and dotted line represents the closed level. (C) Single channel amplitude at -6.7 mV in control and failing rat cardiomyocytes at different locations. Amplitude was unchanged between all groups (control, TT n=19, crest n=4; failing, TT n=11, crest n=13).
Online Figure X. KN-93 blocker reduces LTCC activity in the crest of failing cells. Representative single channel traces at -6.7 mV showing single channel activity in the crests of control, failing, and KN-93-treated failing cardiomyocytes. Dotted line represents the closed level.
Online Figure XI. Effect of KN-92 on failing cardiomyocytes. Percentage of mapped cells that exhibited oscillations are shown for failing cells before and after treatment by KN-92 (n=2 rats for each treated group).
Online Figure XII. Action potential recordings and characteristics from control and failing rat cardiomyocytes. (A) Representative AP trace of a control (left) and a failing rat cardiomyocyte (right) after 5ms threshold pulse at 1Hz stimulation. (B) AP characteristics. Cm indicates membrane capacity. RMP indicates resting membrane potential. APA indicates action potential amplitude. APD indicates action potential duration at 50% and 90%. Values are mean±SEM.
Online Figure XIII. Whole cell L-type calcium current recordings from control and failing rat cardiomyocytes. (A) Whole cell $I_{\text{CaL}}$ current-voltage relationship of control and failing cardiomyocytes (control n=7, failing n=5). (B) Voltage dependence of the time constant of $I_{\text{CaL}}$ inactivation in both control and failing cardiomyocytes from A. * indicates $p<0.05$. 
Simulations of EADs in failing ventricular myocytes as a function of transmural cell type and pacing protocol. (A) Slow pacing at 0.25Hz did not result in EADs in control (left) or failing (right) human epicardial (epi) cells. (B) Pacing human endocardial (endo, far left is control, and second from left is failing) or epi cells (control and failing are second from right and far right, respectively) at 1Hz with a single skipped beat did result in EADs. (C) 1 Hz pacing with two skipped beats resulted in an EAD in failing human endo cells (second from left), but not in the other cases.
Online Tables.

| Patient | Diagnosis | Age | Gender | NYHA | BNP (ng/l) | LV Post Wall Thick. Systole (mm) | LV Post Wall Thick. Diastole (mm) |
|---------|-----------|-----|--------|------|------------|----------------------------------|----------------------------------|
| HF#1    | DCM       | 40  | Male   | IV   | 2839       | 13                               | 9                                |
| HF#2    | DCM       | 33  | Male   | IV   | 624        | 10                               | 8                                |
| HF#3    | DCM       | 57  | Male   | IV   | 1525       | 10                               | 8                                |
| HF#4    | DCM       | 56  | Female | IV   | 1543       | 7                                | 6                                |
| HF#5    | DCM       | 64  | Male   | IV   | 942        | 6                                | 8                                |
| HF#6    | DCM       | 41  | Male   | IV   | 588        | 10                               | 7                                |

Online Table I. Heart failure patients characteristics. HF, heart failure; DCM, dilated cardiomyopathy; NYHA, New York Heart Association functional classification; BNP, B-type Natriuretic Peptide levels.

| Patient | Diagnosis | Age | Gender | Total cells sealed | Occurrence (cells that show LTCC activity) | Pipette resistance (MΩ) |
|---------|-----------|-----|--------|--------------------|------------------------------------------|------------------------|
| NF#1    | MVR       | 78  | Female | 143                | 19%                                      | 32.34 ± 0.85           |
| NF#2    | MVR       | 69  | Male   | 99                 | 26%                                      | 31.93 ± 1.4            |
| NF#3    | MVR       | 66  | Male   | 33                 | 22%                                      | 30.52 ± 1.23           |
| NF#4    | MVR       | 70  | Female | 48                 | 31%                                      | 33.32 ± 1.49           |
| NF#5    | MVR       | 64  | Male   | 48                 | 31%                                      | 25.64 ± 1.16           |

Online Table II. Control patients characteristics. NF, nonfailing; MVR indicates mitral valve replacement procedure.

Online Table III. Patch-clamp single channels seals characteristics. Percentage of successful seals was calculated from the total number of seals tried after the clipping of the pipette. Overall, 7% of the pipettes were discarded after the clipping process and were not used for the analysis. Pipette resistance corresponded to an estimated inner tip diameter range of 260-425 nm for TT and 260-530 nm for Crest recordings.
Online Videos

**Online Video I.** Simulated failing human ventricular movie showing the formation of arrhythmogenic triggers and reentrant arrhythmia. (1Hz pacing with a single skipped beat after two pacing stimulus), after the skipped beat cells failed to repolarize and an endocardial EAD trigger formed. Note that the activation took twice as long to excite the failing ventricles when compare to control.

**Online Video II.** Simulated control human ventricular movie showing organize and regular response to each pacing stimulus (1Hz pacing with a single skipped beat after two pacing stimulus). The skipped beat does not produce any effect on the control ventricle.