SUPPLEMENTAL MATERIAL

Impaired intracellular calcium buffering contributes to the arrhythmogenic substrate in atrial myocytes from patients with atrial fibrillation

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Running title: Impaired Ca^{2+} buffering in persistent AF patients

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Supplemental Methods

All protocols were approved by the Ethics Committee of the University Medical Center Göttingen (No. 10/9/15, 15/2/20 and No. 4/11/18). Informed consent was obtained from all participants and all research was performed in accordance with relevant guidelines and regulations.

Cardiac differentiation of human iPSC

hiPSC cell line UMGi014-C clone 14 (isWT1.14) was derived from the dermal fibroblasts of a healthy male donor (31 years). They were cultured in feeder-free conditions using the integration-free CytoTune iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) with reprogramming factors OCT4, KLF4, SOX2, c-MYC. Previously published pluripotency and karyotype analysis of this line revealed no abnormalities or chromosomal instability.[21]

Incubation for all steps was at 37 °C with 5 % CO_2. Briefly, iPSC were cultured in feeder-free conditions with StemMACS™ iPS-Brew XF (Miltenyi Biotec) until confluency was reached. Mesoderm induction was initiated through 48 hour incubation with 4-5 µmol/L CHIR99021 in a 'Differentiation Medium' containing: RPMI 1640 with Glutamaxx (Thermo Fisher Scientific), 0.5 mg/ml human recombinant albumin and 0.2 mg/ml L-ascorbic acid 2-phosphate (all Sigma-Aldrich). This step is referred to as day 0. After 2 days, cardiac differentiation was started by addition of 5 µmol/L IWP2 (Wnt Antagonist II, Merck) to the differentiation medium for 48 h. For atrial subtype specification, 1 µmol/L retinoic acid (Sigma Aldrich) was added between day 3 and day 6. Cardiac contraction is usually observed on day 7. From day 8 onwards, cells are cultured in a 'culture medium' containing RPMI 1640 with Glutamax, and 2% B27 (both Thermo Fisher Scientific) and maintained every 2-3 days. On day 15, lactate selection was initiated for 7 days to increase the purity of the cell batch in a 'selection medium' of RPMI 1640 without glucose (Thermo Fisher Scientific), 0.5 mg/ml human recombinant albumin, 0.2 mg/ml L-ascorbic acid 2-phosphate, and 4 mM lactate (all Sigma-Aldrich). After 7 days, medium was switched back to culture medium. On day 28, the iPSC-CM were plated on glass coverslips for isolated cell experiments.

Automated Patch Clamp experiments

Measurement of inward rectifier currents in atrial iPSC-CM (day 71) were performed using the automated patch-clamp device, SyncroPatch 384 (Nanion Technologies GmbH) at room temperature.[74] Negative pressure (150-250 mbar) application attained whole-cell configuration. PatchControl 384 (Nanion Technologies GmbH) software was used for data acquisition. Thin borosilicate glass384-well planar chips (1xS-type NPC-384T) were used for all experiments. Recordings were excluded if they showed a seal resistance of <250 MΩ, a peak current of <50 pA, or an R_{series} of >20 MΩ. At the start of an experiment, chips were loaded with 30 µL of a solution containing (in mmol/L): HEPES 10, NaCl 140, KCl 4, glucose 5. Following application of the desired pipette solution to generate electrical contact between external and internal sides of the chip, 20 µL of cell suspension was added to each well. Directly following this, 40 µL of a solution containing (in mmol/L): HEPES 10, NMDG 60, NaCl 80, KCl 4, $CaCl_2$ 10, $MgCl_2$ 1, glucose 5 was temporarily added to aid giga seal formation. 40 µL was then removed from each well and replaced with solution giving a final bath concentration of (in mmol/L): HEPES 10, NMDG 60, NaCl 80, KCl 20, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5. Inward rectifier potassium currents were measured by applying a ramp pulse from −100 to +40 mV (0.5 Hz). Agonist-inducible $I_{K,ACh}$ was stimulated with carbachol (CCh, 2 µmol/L). Inward rectifier currents were defined as those responsive to block with 1 mM $BaCl_2$.[20]

Flow cytometry analysis

For flow cytometric analysis of atrial iPSC-CM (day 51), the cells were dissociated with Trypisn/EDTA (PAN-Biotech) into single cells, fixed with 4% FA at RT for 10 minutes, permeabilized with 90% ice-cold methanol for 15 min followed by a blocking step with 3% BSA and 5% goat serum for 20 min. Cells were then incubated with antibodies against the atrial (MLC2a) and the ventricular (MLC2v) isoform of myosin light chain for 1h at RT. Cells were then incubated with secondary antibody in 3% BSA in PBS at RT for 30 min. Antibodies are listed below in Table S4. Cells were subsequently analyzed using the BD Accuri™ C6 plus system flow cytometer (BD Biosciences). Isotype control for each primary antibody was used as negative control. 30,000 events were acquired and at least 20,000 events were analyzed per sample.

Immunofluorescent staining

Atrial iPSC-CMs were fixed with 4% paraformaldehyde on day 51, permeabilized and blocked with TBS-T including 5% serum for 30 min. Cells were then incubated for overnight at 4°C with the primary antibodies against MLC2a (1:200) and MLC2v (1:200), washed with PBS and incubated with the respective secondary antibody(ies) (1:300) for 30 min. Nuclei were counterstained with DAPI. Epifluorescence images were captured using an Evos FL auto 2 microscope (Life Technologies) and processed with ImageJ software. Antibodies are listed below in Table S4.

Coverslip preparation

Wells containing iPSC-CM were washed twice with DPBS $Ca/Mg^{-/-}$ (Gibco/Thermo Fisher Scientific), and then incubated with Accutase® (Merck) for 10-15 min, until the cells were detached and dissociated. Digestion was stopped with 'recovery medium' containing culture medium supplemented with 1:2000 ROCK inhibitor Y27632 (Stemolecule). Following centrifugation at 4°C at 100 g for 10 minutes, cells were resuspended in recovery medium, counted using a counting chamber (Neubauer improved, Paul Marienfeld GmbH & Co.KG), and plated on glass coverslips (10 mm diameter Menzel coverslip, Thermo Fisher) pre-coated with 1:120 Matrigel® (Corning®) at a density of 10^3 cells/coverslip. iPSC-CM were incubated with recovery medium for 2 days and then switched to culture medium and maintained every 2-3 days. All iPSC-CM measurements were conducted between d35 and d43 unless otherwise indicated.

Simultaneous patch-clamp, intracellular Ca^{2+} and cell-shortening measurements

Atrial cardiac myocytes were transferred to a 37 ± 0.5 °C heated chamber and were superfused with bath solution (in mmol/L: 4-aminopyridine 5; $BaCl_2$ 0.1; $CaCl_2$ 2; Glucose 10; HEPES 10; KCl 4; $MgCl_2$ 1; NaCl 140; Probenecid 2; pH = 7.35 adjusted with NaOH). Simultaneous measurements of membrane currents and intracellular Ca^{2+} were performed under voltage-clamp using the whole cell ruptured patch configuration. Membrane currents were measured and analyzed using pClamp-Software (V 10.7 Molecular Devices). Fluo-3 pentapotassium salt, 0.1 mM (Thermo Scientific) was added to the pipette solution (in

mmol/L: EGTA 0.02; GTP-Tris 0.1; HEPES 10; K-aspartate 92; KCl 48; MgATP 1; Na_2ATP 4; pH = 7.2 adjusted with KOH). Tip resistances of borosilicate glass microelectrodes were 3-7 MΩ. A voltage-clamp protocol using a holding potential of -80 mV and a 100 ms voltage step to 10 mV at 0.5 Hz was employed to activate L-type Ca^{2+} current ($I_{Ca,L}$) and corresponding triggered Ca^{2+} transients. A 100 ms ramp pulse to -40 mV to inactivate the fast Na^+ current was applied before each depolarizing step. Membrane capacitance measurements were acquired and current was expressed as current density (pA/pF).

To quantify intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) Fluo-3 was excited at 488 nm and emitted light (> 520 nm) converted to $[Ca^{2+}]_i$, assuming

$$[Ca^{2+}]_i = k_d \left(\frac{F}{F_{max} - F}\right)$$

where k_d = dissociation constant of Fluo-3 (864 nM), F = Fluo-3 fluorescence, F_{max} = Ca^{2+}-saturated fluorescence obtained at the end of each experiment. Ca^{2+} transients were analyzed by averaging 10 consecutive traces. Sarcoplasmic reticulum (SR) Ca^{2+} content and Ca^{2+} buffering were quantified as previously described by the application of high concentration caffeine (10 mmol/L).[32] Shortening of atrial cardiac myocytes was measured with the aid of an optical cell-edge tracking camera system (MyoCAM-S w CFA300, IonOptix).

SR Ca^{2+} leak quantification

As previously described by Shannon *et al.*,[33] SR Ca^{2+} leak was quantified in atrial IPSC-CM loaded with the calcium-sensitive dye Fluo-3. Briefly, coverslips of IPSC-CM in a 37 ± 0.5 °C heated chamber were electrically stimulated at 1 Hz (IonOptix Myopacer cell stimulator) for 1 min in normal bath solution (in mmol/L: $CaCl_2$ 2; Glucose 10; HEPES 10; KCl 4; $MgCl_2$ 1; NaCl 140; Probenecid 2; pH = 7.35 adjusted with NaOH), followed by perfusion with a Na^+- and Ca^{2+}-free bath solution containing (in mmol/L): EGTA 1, glucose 10, HEPES 10, KCl 4, $LiCl_2$ 140, $MgCl_2$ 1, probenecid 2; pH = 7.35 (adjusted with LiOH) and tetracaine (1 mmol/L, a Ryanodine receptor blocker). The downward deflection of the fluorescence signal is a measure of the SR Ca^{2+} leak.

Optical action potential acquisition

Optical action potentials (AP) were measured by loading the cells with 0.1x VoltageFluor2.1Cl (Fluovolt, Thermo Scientific) for 20 minutes in a bath solution containing (in mmol/L): $CaCl_2$ 2, Glucose 10, HEPES 10, KCl 4, $MgCl_2$ 1, NaCl 140, Probenecid 2; pH = 7.35 adjusted with NaOH. Coverslips containing iPSC-CM were transferred to a 37 ± 0.5 °C heated chamber containing bath solution. Cells were electrically stimulated at 0.5 Hz with two parallel platinum electrodes connected to an external stimulator (IonOptix Myopacer cell stimulator). Stimuli were set to 3-5 ms bipolar pulses with voltages ~ 25 % above the contraction threshold (10-30 V). AP were recorded from isolated masked cells on the stage of an epifluorescence microscope (λ_{Ex} = 470 nm, λ_{Em} = 535 nm), which was optimized for high-speed signal capture with a photomultiplier as previously described.[23,24] Three AP from each cell at every measured frequency were ensemble averaged for offline analysis of AP parameters with Clampfit 10.7 (Molecular Devices).

Immunoblot analysis

Protein isolation and immunoblot analysis were performed as described previously.[29,48] The protein levels of α-actin, cardiac myosin binding protein-C (cMyBP-C), phosphorylated cMyBP-C (P-cMyBP-C, 1:2000), cardiac troponin C (cTnC), calsequestrin (CSQ), cardiac troponin I (cTnI), phosphorylated cTnI (P-TnI), myosin heavy chain 6 (Myh6) and tropomyosin (Tm) were analyzed. Appropriate fluorescence-conjugated donkey anti-rabbit, goat anti-mouse and donkey anti-mouse (all IRDye, LI-COR), and peroxidase-conjugated goat anti-rabbit (Sigma-Aldrich) were used as secondary antibodies. Signals were visualized using a fluorescence and chemiluminescence (Thermo Fisher) imaging system (Odyssey CLx, LI-COR), respectively. Image Studio Software (LI-COR) was used for quantification. Protein expression was normalized to calsequestrin or GAPDH. Antibodies are listed below in Table S5.

Langendorff experiments

Adult mice (C57BL/6J, female, 29-57 weeks) were sacrificed by cervical dislocation. The heart was swiftly removed and placed in bath solution (perfusing solution) with 250 I.E. heparin added (in mmol/L: NaCl 126.7; KCl 5.4; $MgCl_2 \cdot 6H_2O$ 1.05; $CaCl2 \cdot 2H_2O$ 1.8; NaH_2PO_4 0.42;

$NaHCO_3$ 22). Hearts were retrogradely perfused on a Langendorff system (2-4.5 mL/min). All experiments were performed at 37 °C, using a warmed organ chamber and warmed solutions. Upon commencement of perfusion, a pacing electrode and three measurement electrodes (electrogram) were placed on the right and left atrium and the apex of the heart. A hypodermic needle (0.4 mm) was inserted into the left ventricle in order to alleviate pressure. Additionally, perfusing solution was also applied externally to the heart (in drip form) throughout the investigation, to prevent the epicardium drying out. Potassium concentration in the perfusing solution was decreased step-wise and the heart was perfused in the absence of stimulation before each round of measurements. At each concentration of potassium, a series of measurements were performed (see also Supplemental Figure S5A):

1. S1-S2 protocol for measurement of absolute refractory period (3 times)
2. Step burst pacing (increasing pacing frequencies, 30 seconds at each frequency): 400 bpm; 700 bpm; 1200 bpm; 1600 bpm; 2000 bpm; 2300 bpm; 2600 bpm; 3000 bpm; 3200 bpm; 3400 bpm; 3600 bpm; 4000 bpm; 4500 bpm
3. Burst pacing (8 V): 6000 bpm for 5 sec (3 times, separated by 30 sec pauses in stimulation)
4. Burst pacing (9 V): 6000 bpm for 5 sec (3 times, separated by 30 sec pauses in stimulation)

The above measurement series was carried out a total of 7 times (one measurement "round") with 5.4 mmol/L KCl in the perfusing solution (15 min total measurement round time). 5 µmol/L blebbistatin was then added to the perfusing solution and the measurement round performed twice. This was repeated with 3.7 mmol/L KCl and 5 µmol/L blebbistatin in the perfusate, 2.0 mmol/L KCl and 5 µmol/L blebbistatin in the perfusing solution and, finally, 2.0 mmol/L KCl with both 5 µmol/L blebbistatin and 300 µmol/L diazoxide in the perfusing solution. For control experiments, the aforementioned potassium (and diazoxide) concentrations were used in the perfusing solution, but blebbistatin was omitted. The data were analyzed for arrhythmic activity (inducibility and episode duration). In addition, absolute refractory period was quantified.

Skinned fiber preparation and force measurements

Skinned fibers were prepared as described previously.[27] Right atrial appendages were transferred to an ice-cooled vial (4 °C), filled with modified cardioplegic solution (Krebs-Henseleit solution, in mmol/L: NaCl 118.07, Dextrose 11.1; KCL 4.7; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; $CaCl_2 \cdot 2H_2O$ 1.8) containing BDM (30 mmol/L). In the laboratory, the tissue was placed in a dish, filled with ice-cooled (4 °C) preparation solution (in mmol/L: Imidazole 68.08; Sodium azide 65.01; Ethyleneglycol tetraacetic acid 380.4; Dithioerythritol 154.3; $MgCl_2 \cdot 6H_2O$ 203.3; ATP 605.2). Resected muscle bundles were placed in a 1 % Triton X-100 solution for 24 h at 4°C on a shaking device to permeabilize fiber membranes. After this skinning process, the RA muscle bundles were transferred to a separate ice-cooled (4 °C) dish, filled with preparation solution and single RA muscle strips were prepared (size of 2–2.5 mm × 0.3 mm) under the microscope (x10, magnification, Leica S6D stereomicroscope; Leica Microsystems GmbH, Wetzlar, Germany).

A muscle investigation system (Gradient Program; Scientific Instruments, Heidelberg, Germany) was used to expose the RA fibers to a gradual increase in the calcium concentration for force measurements. The RA fibers were fixed in a perfusion chamber and incubated with a relaxation buffer (in mmol/L: Imidazole 68.08; Creatine phosphate 327.2; Sodium azide 65.01, Ethyleneglycol tetraacetic acid 380.4, $MgCl_2$ 203.3; Dithioerythritol 154.2; ATP 605.2; creatine kinase 400 U/ml). By adding $CaCl_2$ (147.02 mmol/L) to the relaxation solution, a pCa-force curve was created. Specially designed software (Gradient Program; Scientific Instruments) was used to calculate the necessary amount of calcium to achieve a stepwise increase in the calcium concentration, according to an equation proposed by Fabiato and Fabiato.[27] The pCa starts with 6.5 ≤ 4.0 in 0.5 increments. For each patient, a set of three RA fibers underwent calcium-induced force measurements. The mean of these three measurements was determined.

Isolation of human myofilament proteins and quantification of phosphorylation

Approximately 20-30 mg patient atrial tissue (frozen in liquid nitrogen) was homogenized in 1 ml relaxing buffer (in mmol/L: KCl 75; Imidazole pH 7.2 10; $MgCl_2$ 2; EGTA 2; EDTA 10; Sodium azide 1; phosphocreatine 4; ATP 1; 2,3-Butanedione monoximine 50; DTT 1; Benzamidine-HCl

1) with 1% (vol/vol) Triton X-100, followed by centrifugation at 11,000 g for 10 min (supernatant discarded). The pellet was then homogenized in 800 µl rigor buffer (in mmol/L: KCl 75; Imidazole pH 7.2 10; $MgCl_2$ 2; EGTA 2; EDTA 10; Sodium azide 1) with 1% (vol/vol) Triton X-100 and centrifuged at 11,000 g for 10 min and the supernatant discarded. This was repeated twice. The pellet was washed in 800 µl rigor buffer without Triton X-100, followed by centrifugation at 11,000 g for 10 min and the supernatant discarded. Finally the pellet was washed again in 1 ml rigor buffer without Triton X-100. DC™ Protein Assay kit was used to quantify myofilament protein concentration. The remainder was centrifuged at 11,000 g for 10 min (supernatant discarded) and washed in 1 ml wash buffer (in mmol/L: KCl 60; MOPS pH 7.0 20; $MgCl_2$ 2), followed by centrifugation at 11,000 g for 10 min (supernatant discarded). The washing step was repeated, followed by solubilization in sample buffer (urea 8 mol/L; Thiourea 2 mol/L; Tris·HCl pH 6.8 50 mmol/L; DTT 75 mmol/L; SDS 3%; Bromophenol blue 0.05%), to give an end protein concentration of 4 µg/µl. After centrifugation at 11,000 g for 10 min, the supernatant, containing myofilament proteins, was collected. All steps, as described previously,[28] were performed at 4 °C and all buffers used for the myofibrillar preparation contained protease and phosphatase inhibitors (PMSF 0.25 mM; Leupeptin 1.25 mg/l; Pepstatin 1.25 mg/l; NaF 50 mM; and Na_3VO_4 0.25 mM).

SDS-PAGE was carried out in a BioRad system (Mini-PROTEAN Tetra cell); samples (50 µg protein) were loaded on to a BioRad Mini Protean TGX 10% Gel which was run at 120 V for 50 min in running buffer (Tris 125 mmol/L, glycine 192 mmol/L, and SDS 0.1 %). The gel was stained with Pro-Q Diamond gel stain (Molecular Probes) to detect phosphoproteins, fixed in 100 ml of 50 % methanol with 10 % acetic acid for 30 min (repeated once) and then washed three times (10 min each) in 100 ml water. The gel was then stained in 60 ml Pro-Q™ Diamond stain for 1 h at 4 °C and then destained three times (30 min each) in 80-100 mL Pro-Q™ Diamond destaining solution, followed by two wash steps (5 min each) in 100 mL water. Imaging of the gel was performed with a ChemiDoc Imaging System (BioRad, USA) (555 nm excitation, 580 nm emission).

To detect total protein, the gel was stained in 60 mL SYPRO™ Ruby gel stain (Molecular Probes) overnight and then washed three times (30 min each) in 100 mL wash buffer (10 % methanol with 7 % acetic acid), followed by at least two washes (five min each) in water. Imaging of the

gel was performed with a ChemiDoc Imaging System (BioRad, USA) (280 nm excitation, 450 and 610 nm emission). Phosphorylation levels were measured by normalizing ProQ™ Diamond signal to SYPRO™ signal. Image Lab version 6.1 (BioRad, USA) was used to determine quantitative optical densities of the bands from Pro-Q Diamond- and SYPRO Ruby-stained gels.

Computational modelling of reduced Ca^{2+} buffering

We employed a previously published human atrial cardiomyocyte model with spatial Ca^{2+} handling and stochastic RyR2 gating[34] to simulate systolic and spontaneous Ca^{2+} events. The published model was paced for 100 beats at 0.5 Hz to stabilize intracellular ion concentrations. Subsequently, the model was paced for 6 beats followed by a 30-s follow-up period in the presence of either normal Ca^{2+} buffering (baseline) or reduced Ca^{2+} buffering and systolic Ca^{2+}-transient, spontaneous Ca^{2+} wave (SCaW) and delayed afterdepolarization (DAD) parameters were quantified. Reduced Ca^{2+} buffering was simulated as a 20% reduction in total high and low affinity troponin buffer sites (Bmax_TnChigh and Bmax_TnClow parameters) and a 25% reduction in the time constants of Ca^{2+} diffusion between segments and domains ($\tau_{diff,seg,i}$, $\tau_{diff,dom,i}$, $\tau_{diff,seg,srs}$, $\tau_{diff,dom,srs}$). Model code is available on https://github.com/HeijmanLab/

Supplemental Tables

Table S1. Characteristics of patients used for electrophysiological experiments

	Ctrl	persAF
Patients, n	16	13
Sex, male/female	14/2	8/5
Age, y	65.2 ± 2.3	65.1 ± 4.1
Body mass index, kg/m^2	28.3 ± 1.2	28.2 ± 1.2
CAD, n	14	8
MVD/AVD, n	2	4
CAD+MVD/AVD, n	0	1
Hypertension, n	14	8
Diabetes, n	2	2
Hyperlipidemia, n	7	4
LVEF, %	50.4 ± 2.9	51.4 ± 2.7
Digitalis, n	0	1
ACE inhibitors, n	10	7
AT1 blockers, n	5	5
β-Blockers, n	13	10
Dihydropyridines, n	3	3
Diuretics, n	6	7
Nitrates, n	1	1
Lipid-lowering drugs, n	13	8

ACE, angiotensin-converting enzyme; AT, angiotensin receptor; CAD, coronary artery disease; LVEF, left ventricular ejection fraction; MVD/AVD, mitral/aortic valve disease. Continuous data are expressed as mean ± SEM. Comparison was made using Student's t-test and Fisher's exact test for continuous and categorical data, respectively.

Table S2. Characteristics of patients used for protein biochemistry experiments

	Ctrl	persAF
Patients, n	12	12
Sex, male/female	9/3	5/7
Age, y	63.5 ± 2.9	74.8 ± 1.7**
Body mass index, kg/m^2	27.9 ± 0.7	28.0 ± 1.3
CAD, n	11	6
MVD/AVD, n	1	5
CAD+MVD/AVD, n	0	1
Hypertension, n	10	12
Diabetes, n	3	3
Hyperlipidemia, n	4	7
LVEF, %	51.7 ± 4.3	50.6 ± 3.2
Digitalis, n	0	1
ACE inhibitors, n	4	4
AT1 blockers, n	6	6
β-Blockers, n	10	10
Dihydropyridines, n	2	0
Diuretics, n	4	7
Nitrates, n	0	0
Lipid-lowering drugs, n	11	7

ACE, angiotensin-converting enzyme; AT, angiotensin receptor; CAD, coronary artery disease; LVEF, left ventricular ejection fraction; MVD/AVD, mitral/aortic valve disease. Continuous data are expressed as mean ± SEM. Comparison was made using Student's t-test and Fisher's exact test for continuous and categorical data, respectively. $^{**}P < 0.01$.

Table S3. Characteristics of patients used for protein biochemistry experiments (Figure S5)

	Ctrl	persAF
Patients, n	14	14
Sex, male/female	13/1	10/4
Age, y	61.6 ± 2.6	70.6 ± 2.1*
Body mass index, kg/m^2	29.1 ± 1.7	27.4 ± 1.2
CAD, n	11	8
MVD/AVD, n	2	4
CAD+MVD/AVD, n	1	2
Hypertension, n	11	12
Diabetes, n	3	7
Hyperlipidemia, n	8	6
LVEF, %	46.7 ± 3.4	52.1 ± 3.4
Digitalis, n	0	1
ACE inhibitors, n	4	5
AT1 blockers, n	3	6
β-Blockers, n	10	14
Dihydropyridines, n	3	4
Diuretics, n	3	6
Nitrates, n	0	0
Lipid-lowering drugs, n	13	8

ACE, angiotensin-converting enzyme; AT, angiotensin receptor; CAD, coronary artery disease; LVEF, left ventricular ejection fraction; MVD/AVD, mitral/aortic valve disease. Continuous data are expressed as mean ± SEM. Comparison was made using Student's t-test and Fisher's exact test for continuous and categorical data, respectively. *$P < 0.05$.

Table S4. Antibodies used for flow cytometry analysis and immunofluorescent staining

Antibody	Concentration	Company	Identifier
MLC2a	1:200	Synaptic Systems	311 011
MLC2v	1:100	Proteintech	10906-1-AP
Mouse IgG2b	1:200	Dako	X0944
Rabbit IgG	1:1600	Abcam	ab37415
Donkey Anti Mouse IgG - Cy3	1:1000	JIR	715-165-150
Goat Anti Rabbit IgG - Cy2	1:1000	JIR	111-225-144

Table S5. Antibodies used for immunoblot analysis

Antibody	Concentration	Company	Identifier
α-actin	1:10000	Invitrogen	PA5-21396
cMyBP-C	1:5000	Abcam	108522
P-cMyBP-C	1:2000	*	
cTnC	1:1000	HyTest	4T27cc
cTNC	1:200	Santa Cruz	sc-52263
cTnI	1:1000	Cell Signalling Technology	4004
P-TnI	1:1000	Cell Signalling Technology	4002
Myh6	1:10000	Proteintech	22281-1-AP
Tm	1:200	Abcam	ab7785
CaMKII	1:1000	BD Biosciences	611292
pT286-CaMKII-	1:1000	Abcam	ab32678
Junctophilin-2	1:500	Santa cruz	sc-398125
RyR2	1:1000	Thermo Fisher	MA3-916
pS2808-RyR2	1:1000	Badrilla	A010-30
pS2814-RyR2	1:500	Badrilla	A010-31
CSQ	1:1000	Thermo Fisher	PA1-913
GAPDH	1:80,000	Biotrend	MBS6251847
GAPDH	1:1000	Santa Cruz	sc-47724
IRDye Donkey Anti-rabbit IgG (H+L)	1:10000	LI-COR	926-32213
IRDye Donkey Anti-mouse IgG (H+L)	1:10000	LI-COR	926-32212
IRDye Goat Anti-mouse IgG (H+L)	1:10000	LI-COR	926-32210
IRDye Donkey Anti-mouse IgG (H+L)	1:10000	LI-COR	926-68072
Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L)	1:20000	Jackson ImmunoResearch	111-035-144

* Kindly provided by Prof. Lucie Carrier, Hamburg, Germany.

Supplemental Figures

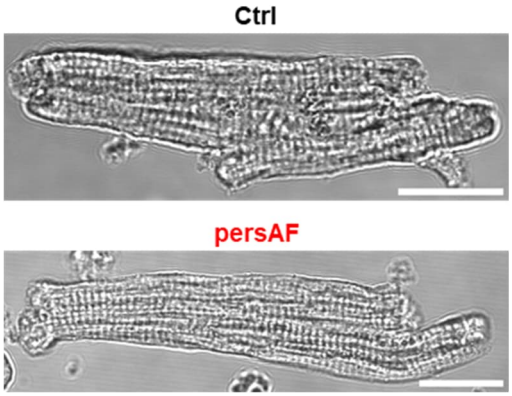

Figure S1. Representative images of human atrial myocytes. Brightfield images of human atrial myocytes isolated from a sinus rhythm (Ctrl) and a persistent atrial fibrillation (persAF) patient. Scale bar = 20 µm.

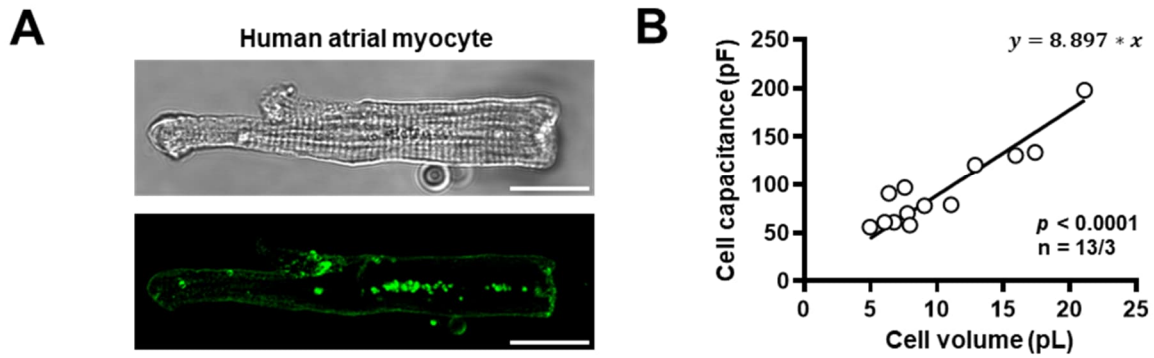

Figure S2. Determination of the relationship between cell capacitance and cell volume of human atrial myocytes. A, Brightfield (top) and confocal z stack (bottom) images of an atrial myocyte obtained from a sinus rhythm patient stained with di-4-ANEPPS. B, Cell capacitance plotted against corresponding estimated cell volume determined from confocal z stack images during simultaneous patch-clamp and confocal line scan measurements of human atrial myocytes. The solid line represents a linear regression line (solid line) fitted to the data points. The coefficient of determination (R^2) and Pearson's correlation coefficient (r) are 0.8300 and 0.9261, indicating a robust linear relationship between capacitance and volume. n/N = number of myocytes/patients. Scale bar = 20 µm.

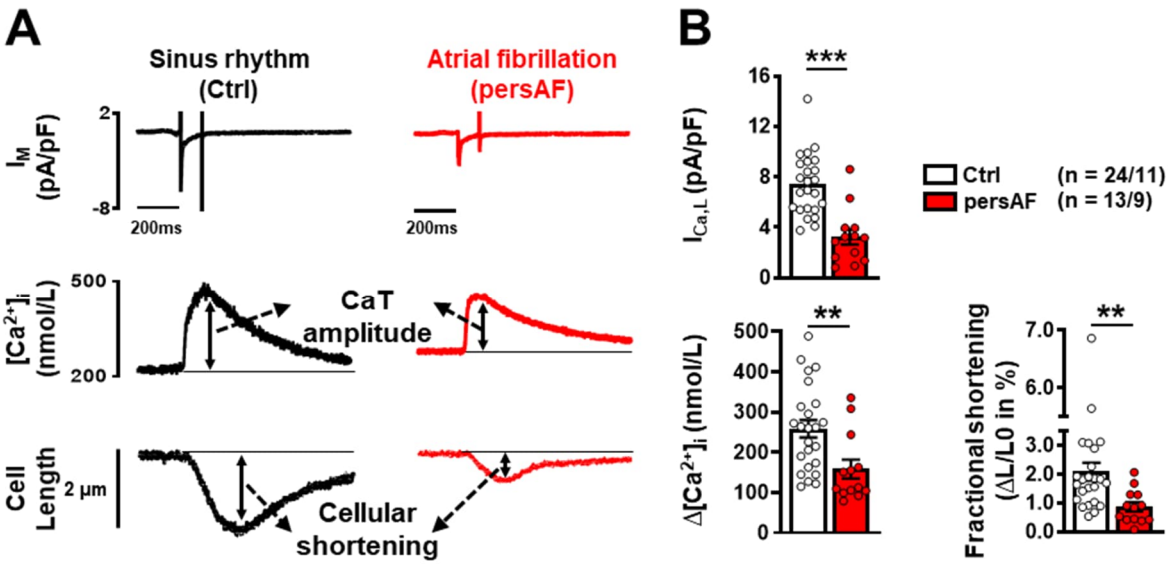

Figure S3. Combined measurement of L-type calcium current ($I_{Ca,L}$), Ca^{2+} transient (CaT) and cell shortening in atrial myocytes from sinus rhythm (Ctrl) and persistent atrial fibrillation (persAF) patients. A, Representative simultaneous recordings of $I_{Ca,L}$ (top), CaT (middle) and cell shortening (bottom) in atrial myocytes from Ctrl and persAF patients using a voltage-clamp protocol (0.5 Hz, insert). B, Mean ± SEM of peak $I_{Ca,L}$ (top left), CaT amplitude (bottom left) and fractional shortening (bottom right) of atrial myocytes from Ctrl and persAF patients. **p<0.01, ***p<0.001 vs Ctrl. n/N = number of myocytes/patients. Normality of data was determined by Shapiro-Wilk test, while comparison was made using Student's t-test and Mann Whitney's U-test for normally and non-normally distributed data, respectively. Ctrl, control. Representative traces of $I_{Ca,L}$ and CaT in this figure are the same traces used in Figure 1 of the main manuscript.

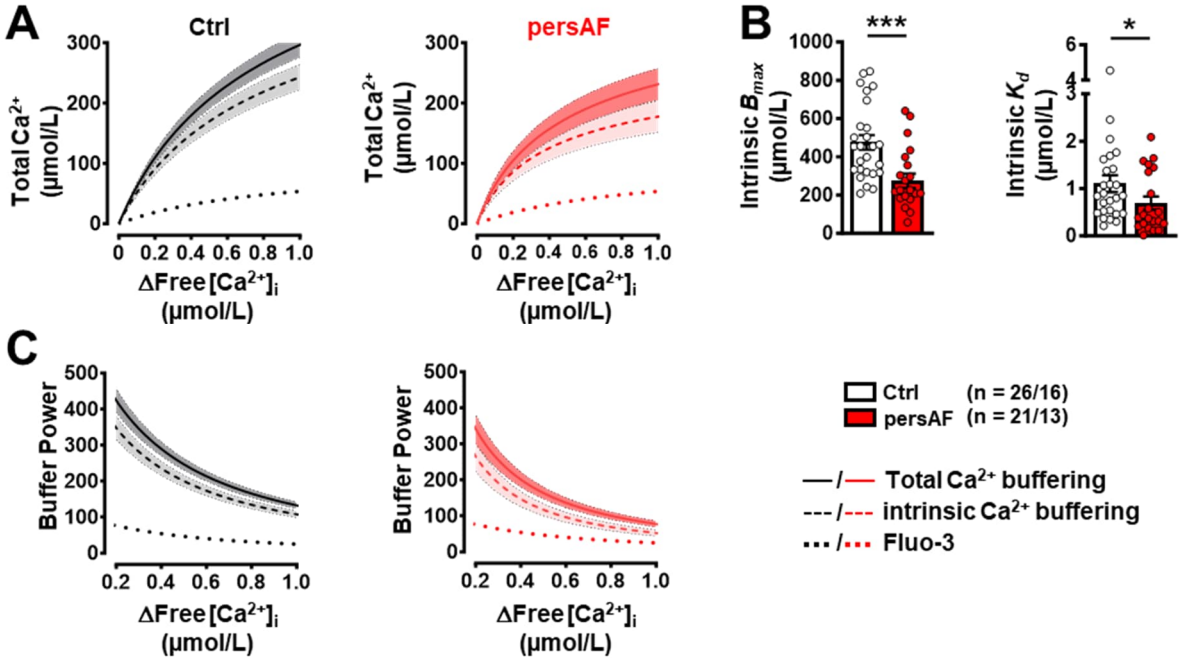

Figure S4. Buffering properties of intrinsic buffers and Fluo-3 in atrial myocytes from sinus rhythm (Ctrl) and persAF patients. A, Mean (line) ± SEM (shaded) derived buffering curves depicting the contribution of intrinsic buffers and the calcium-sensitive dye Fluo-3, to the total cytosolic buffering of Ca^{2+} in Ctrl (left) and persAF (right). B, Mean ± SEM maximum buffering capacity (B_{max}, left) and dissociation constant (K_d, right) of intrinsic buffers, determined by subtracting the estimated Fluo-3 buffer curve from the total buffer curve. C, Mean (line) ± SEM (shaded) calculated buffer power curves of total, intrinsic and Fluo-3 as a function of free $[Ca^{2+}]_i$ in Ctrl (left) and persAF (right). ***P < 0.001, *P < 0.05 vs. Ctrl. n/N = number of myocytes/patients. Normality of data was determined by Shapiro-Wilk test, while comparison was made using Mann Whitney's U-test. Ctrl, control; persAF, persistent atrial fibrillation.

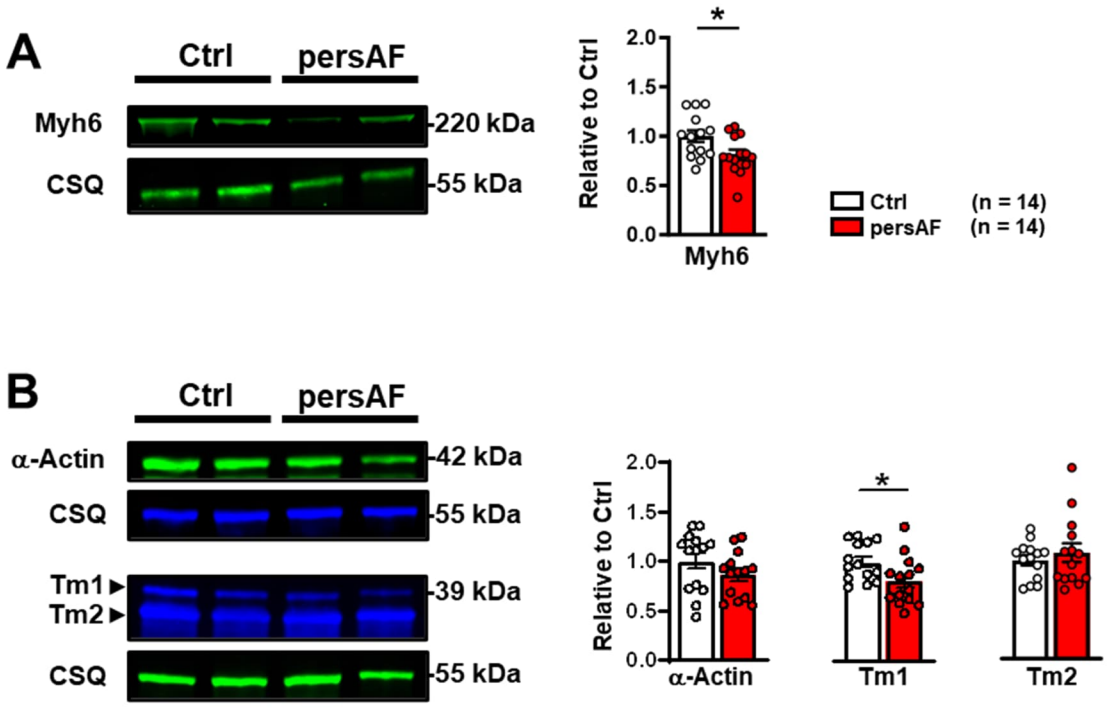

Figure S5. Myofilament protein expression in Ctrl and persAF. A, Immunoblots (left) and quantification (right) of atrial myosin heavy chain isoform 6 (Myh6) in atrial samples from Ctrl and persAF patients, normalized to calsequestrin (CSQ). B, Immunoblots (left) and quantification (right) of α-actin, tropomyosin isoform 1 (Tm1) and 2 (Tm2) in atrial samples from Ctrl and persAF patients, normalized to calsequestrin (CSQ). Data are presented as Mean ± SEM. *P < 0.05 vs. Ctrl. n = number of patients. Normality of data was determined by Shapiro-Wilk test, while comparison was made using Student's t-test with Welch's correction. Ctrl, control; persAF, persistent atrial fibrillation.

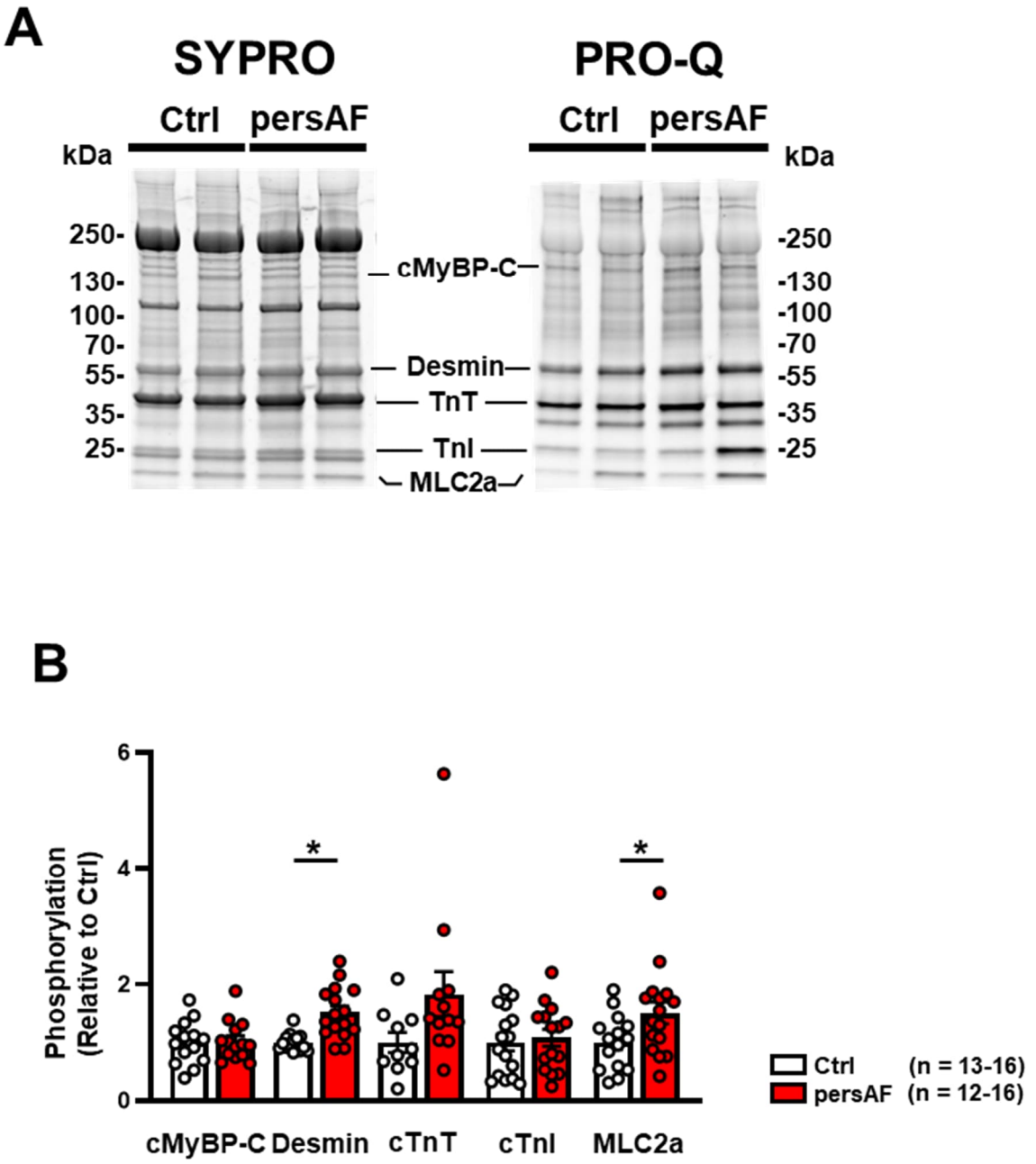

Figure S6. Phosphorylation of myofilament proteins. A, Gels stained with SYPRO (left) and Pro-Q Diamond (right). B, Mean ± SEM phosphorylation of myofilament proteins, relative to Ctrl. *P < 0.05 vs. Ctrl. n = patients. Normality of data was determined by Shapiro-Wilk test, while comparison was made using Student's t-test with Welch's correction and Mann Whtney's U-test for normally and non-normally distributed data, respectively. MLC2a, myosin light chain 2a (atrial isoform); Ctrl, control; cMyBP-C, cardiac myosin binding protein C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; persAF, persistent atrial fibrillation.

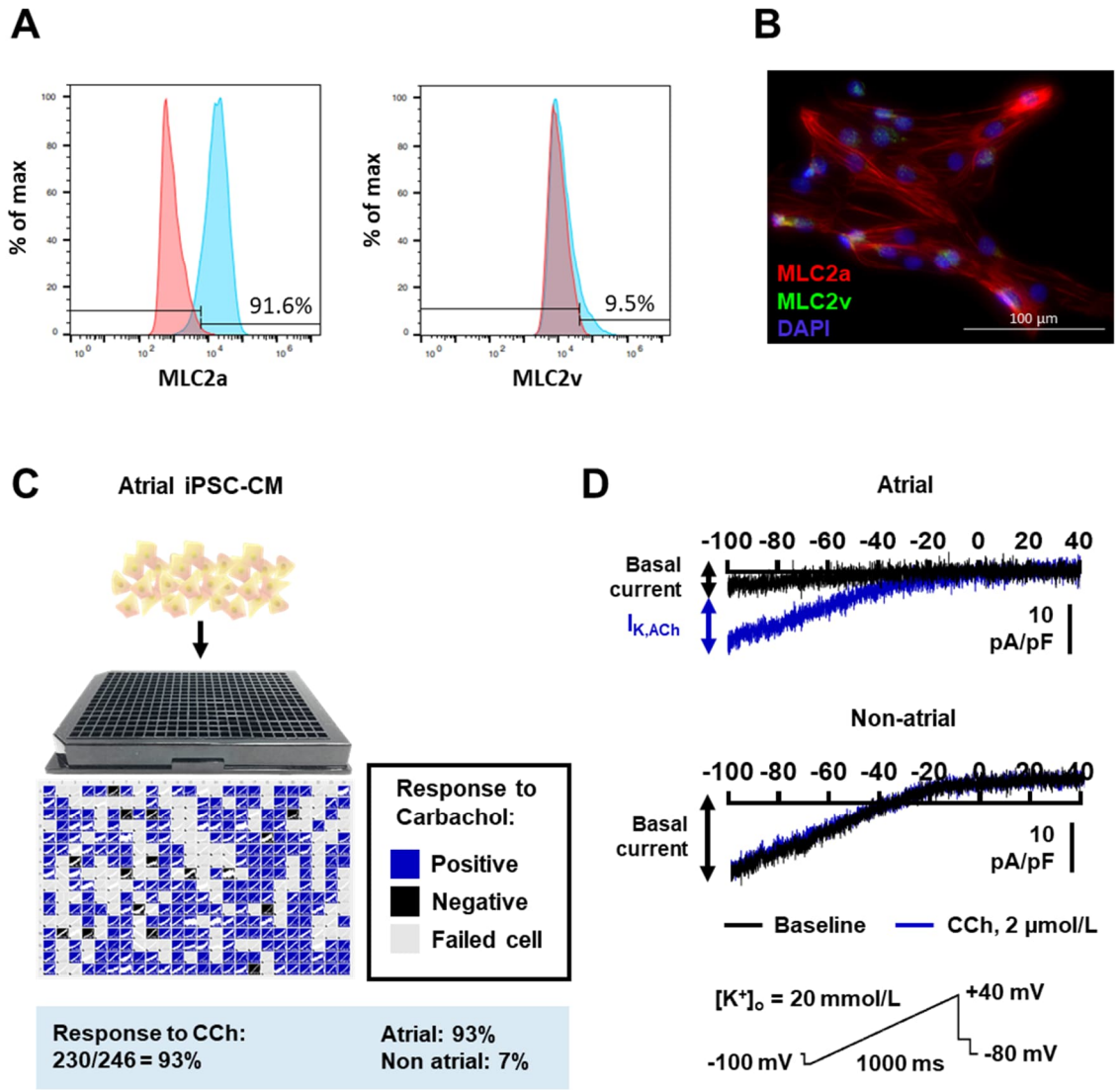

Figure S7. Structural and functional screening for atrial iPSC-CM specificity. A, Representative flow cytometry analysis of iPSC-aCM staining for the atrial isoform (MLC2a, left) or the ventricular isoform (MLC2v, right) of myosin light chain. Blue peaks represent the isotype control. B, Immunofluorescent staining of atrial iPSC-CM to reveal subtype specifc proteins. C, Atrial iPSC-CM distributed across a Nanion recording chip (thin borosilicate glass, single-aperture, low resistance 384-well planar fixed-well, 1xS-type NPC-384T; top) used for simultaneous patch clamp experiments with a high throughput automated patch clamp device (Syncropatch 384, Nanion Technologies GmbH). Upon measurement of inward rectifier currents at -100 mV, a response to 2 µmol/L carbachol (CCh) represents the acetylcholine activated inwardly rectifying potassium current ($I_{K,ACh}$) which is only expressed in atrial cells, indicating an atrial specific phenotype. A screenshot of analysis software (DataControl 384, Nanion Technologies GmbH) shows the number of atrial specific cells identified. Global results from a single cellular batch are calculated (bottom). D, Representative examples of atrial cells expressing $I_{K,ACh}$ (top) and non atrial cells showing basal inward rectifier current, but no $I_{K,ACh}$ (middle). Currents were activated with a ramp pulse from −100 to +40 mV at 0.5 Hz (bottom).

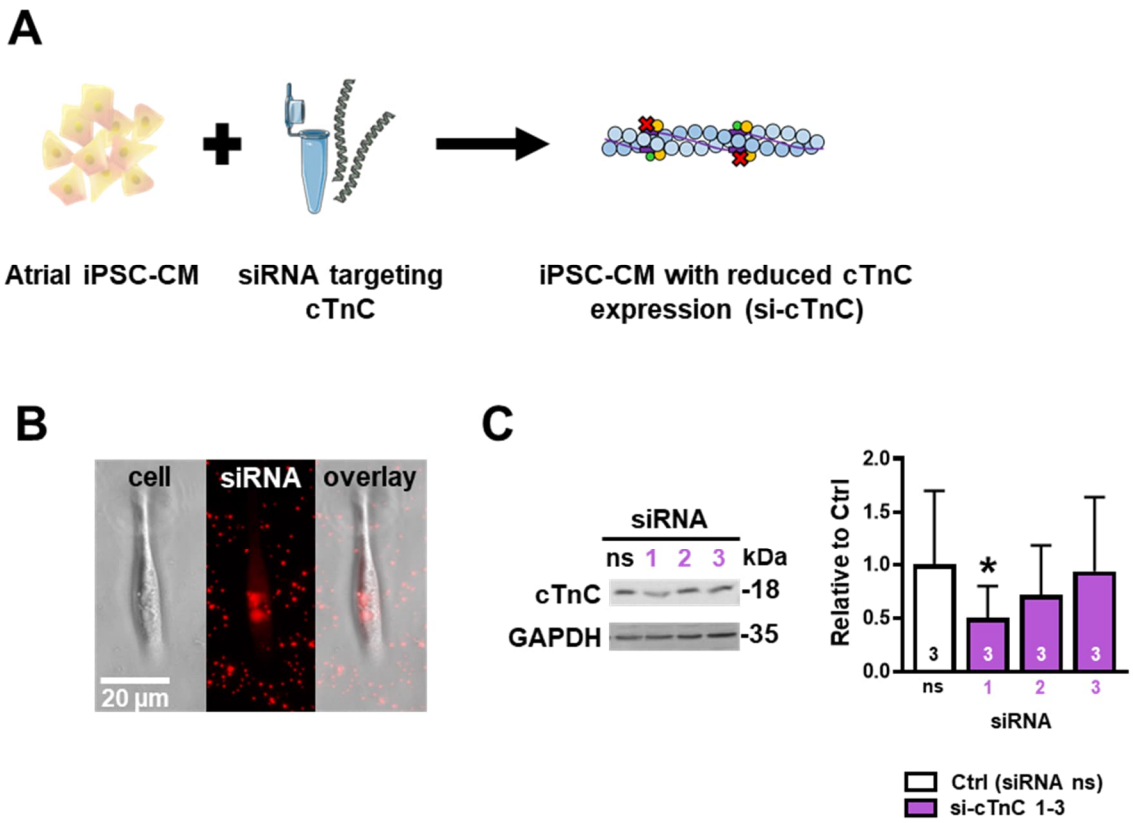

Figure S8. Establishment of an atrial iPSC-CM model with reduced cTnC expression. A, Atrial iPSC-CM were treated with siRNA targeting cTnC, to reduce cTnC expression. B, Treatment of atrial iPSC-CM (left) with red fluorescent control siRNA confirmed efficient transfection (middle and right). C, Immunoblotting of cTnC protein levels in iPSC-CM transfected with three different siRNAs targeting cTnC (si-cTnC) and a non-targeting control siRNA (Ctrl, siRNA ns). siRNA ns (ID: 4390843), siRNA 1 (ID: s14273), siRNA 2 (ID: s224742) and siRNA 3 (ID: s224743) were all obtained from ThermoFisher Scientific. Data presented as Mean ± SEM. *$P < 0.05$ vs. Ctrl. n = rounds. Comparison was made using Friedman test with Dunn's multiple comparison. cTnC, cardiac troponin C; iPSC-CM, induced pluripotent stem cell-derived cardiac myocyte; ns, non-silencing; small interfering RNA, siRNA.

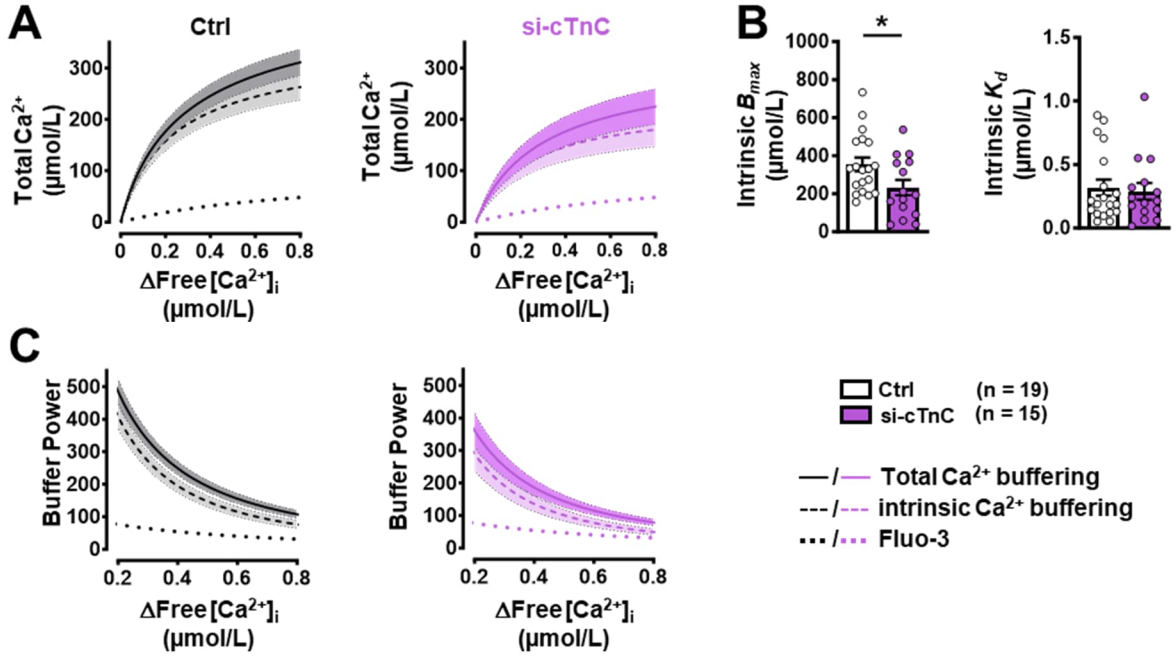

Figure S9. Buffering properties of intrinsic buffers and Fluo-3 in atrial iPSC-CM with normal (Ctrl) and reduced (si-cTnC) cTnC levels. A, Mean (line) ± SEM (shaded) derived buffering curves depicting the contribution of intrinsic buffers and the calcium-sensitive dye Fluo-3, to the total cytosolic buffering of Ca^{2+} in Ctrl (left) and si-cTnC (right). B, Mean ± SEM maximum buffering capacity (B_{max}, left) and dissociation constant (K_d, right) of intrinsic buffers, determined by subtracting the estimated Fluo-3 buffer curve from the total buffer curve. C, Mean (line) ± SEM (shaded) calculated buffer power curves of total, intrinsic and Fluo-3 as a function of free $[Ca^{2+}]_i$ in Ctrl (left) and si-cTnC (right). Normality of data was determined by Shapiro-Wilk test, while comparison was made using Mann-Whitney's U-test. *P < 0.05 vs. Ctrl. n = number of myocytes (2-4 differentiations). Ctrl, control; cTnC, cardiac troponin C; CaT, Ca^{2+} transient; iPSC-CM, induced pluripotent stem cell-derived cardiac myocyte; ns, non-silencing; siRNA, small interfering RNA.

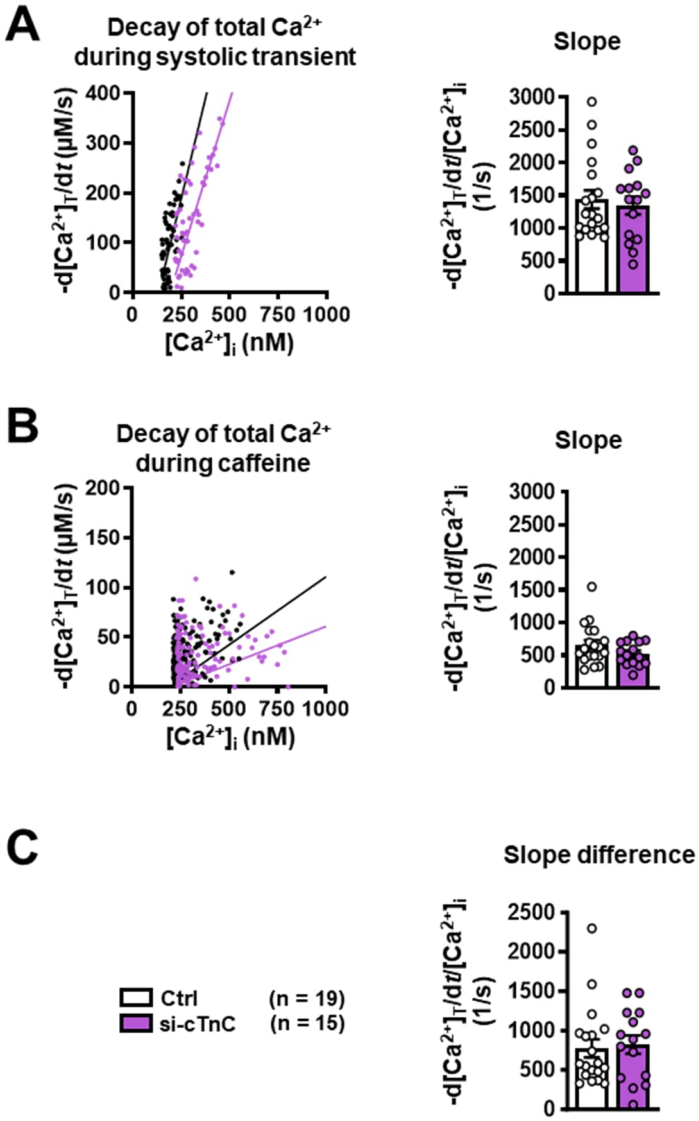

Figure S10. Quantification of decay of total Ca^{2+} in atrial iPSC-CM with normal (Ctrl) and reduced cTnC levels (si-cTnC). A, Representative rate of decay of total Ca^{2+} ($-d[Ca^{2+}]_{total}/dt$) plotted against free $[Ca^{2+}]_i$ (left) and slope of $-d[Ca^{2+}]_{total}/dt$ plotted against $[Ca^{2+}]_i$ (right). B, Representative rate of decay of total Ca^{2+} during caffeine-induced Ca^{2+} transient plotted against the corresponding free $[Ca^{2+}]_i$ (left) and slope of $-d[Ca^{2+}]_{total}/dt$ during caffeine plotted against corresponding $[Ca^{2+}]_i$ (right). C, Difference between slopes in A and B, indicating unaltered $[Ca^{2+}]_i$ dependence of SERCA-mediated Ca^{2+} removal. SERCA contribution to decay of systolic Ca^{2+}: 53.0 ± 3.5 % (Ctrl) and 55.6 ± 4.5 % (si-cTnC). n = number of myocytes (2-4 differentiations). Normality of data was determined by Shapiro-Wilk test, while comparison was made using Student's t-test with Welch's correction and Mann Whitney's U-test for normally and non-normally distributed data, respectively. Ctrl, control; cTnC, cardiac troponin C; iPSC-CM, induced pluripotent stem cell-derived cardiac myocyte.

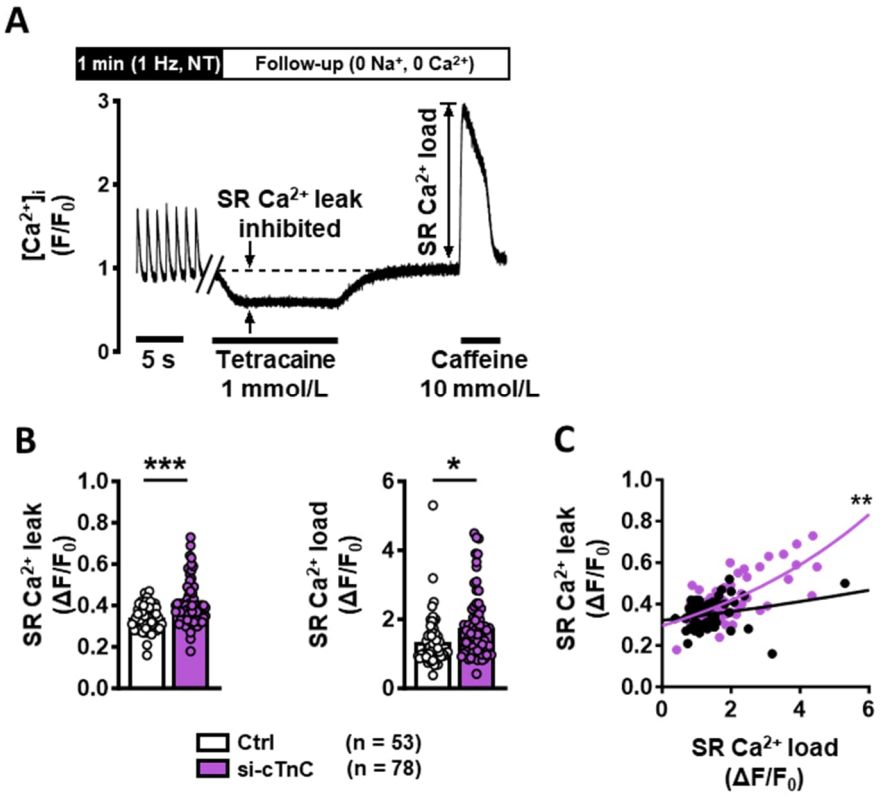

Figure S11. SR Ca^{2+} leak quantification in atrial iPSC-CM with normal (Ctrl) and reduced (si-cTnC) cTnC levels. A, Representative normalized trace illustrating the protocol for quantifying SR Ca^{2+} leak and SR Ca^{2+} content in atrial iPSC-CM using the tetracaine protocol developed by Shannon *et al.*[33] B, Mean ± SEM total SR Ca^{2+} leak (left) in Ctrl (siRNA ns) and si-cTnC (siRNA cTnC) atrial iPSC-CM and SR Ca^{2+} load (right) quantified using caffeine-induced Ca^{2+} transient amplitude. C, SR Ca^{2+} leak plotted against SR Ca^{2+}-load. The curves represent the fitting of exponential regression to the dataset. ***P < 0.001, **P < 0.01, *P < 0.05 vs. Ctrl. n = number of myocytes (2-3 differentiations). Comparison was made using unpaired Student's t-test. Ctrl, control; cTnC, cardiac troponin C; iPSC-CM, induced pluripotent stem cell-derived cardiac myocyte; ns, non-silencing; siRNA, small interfering RNA; SR, sarcoplasmic reticulum; NT, normal Tyrode.

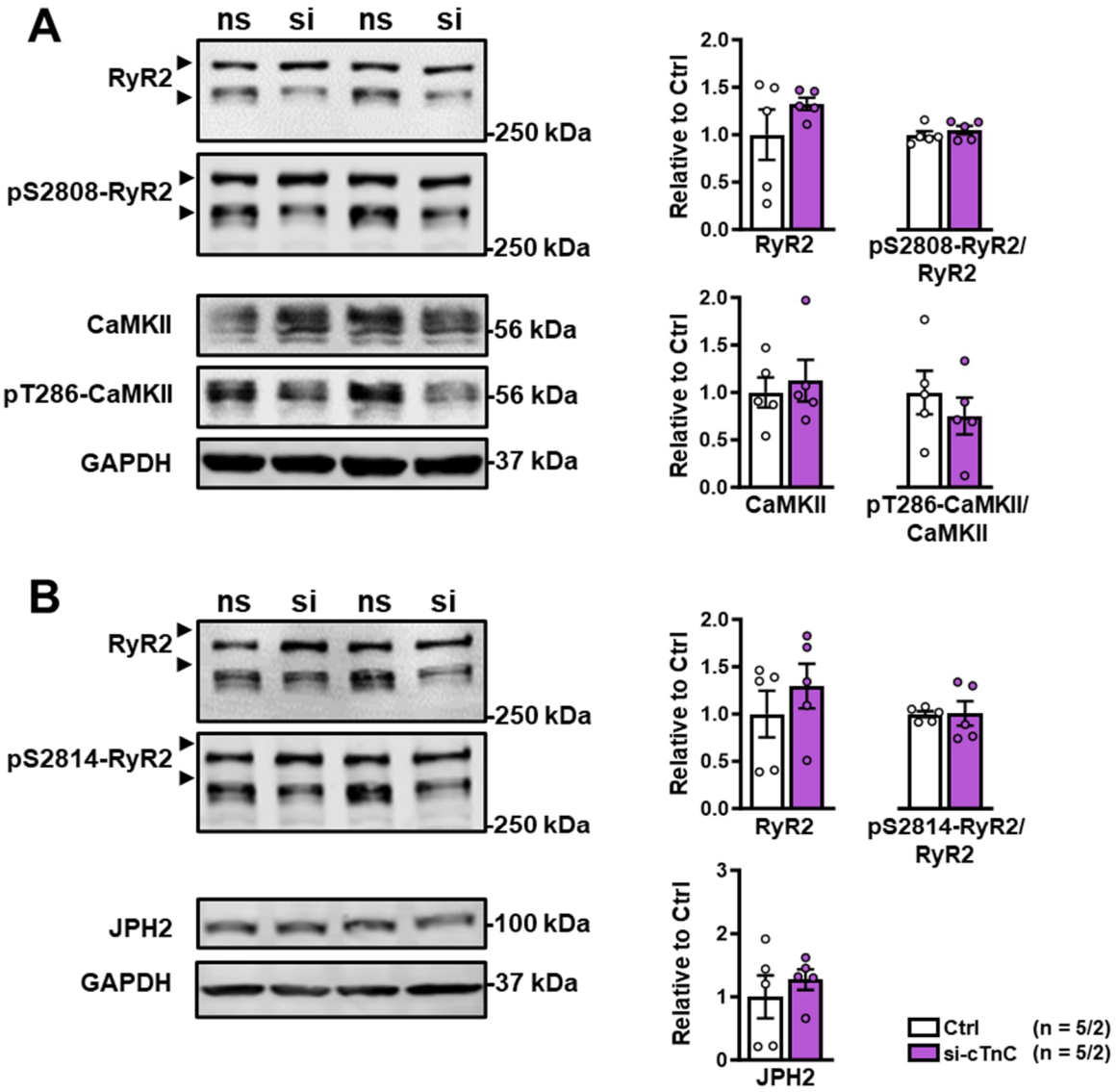

Figure S12. Protein expression and phosphorylation status of RyR2 and its regulatory proteins in atrial iPSC-CM with normal (Ctrl) and reduced (si-cTnC) cTnC levels. A, Immunoblots (left) and quantification (right) of the protein expression of ryanodine receptor 2 (RyR2) and Ca^{2+} calmodulin protein kinase II (CaMKII) as well as their phosphorylation at Serine 2808 (pS2808-RyR2) and tyrosine 286 (pT286-CaMKII), respectively in in Ctrl (siRNA ns) and si-cTnC (siRNA cTnC) atrial iPSC-CM. B, Immunoblots (left) and quantification (right) of the protein expression RyR2 and its phosphorylation at serine 2814 (pS2814-RyR2, alongside the protein expression of Junctophilin-2 (JPH2). RYR2, CaMKII and JPH2 were normalized to GAPDH. pS2808-RyR2 and pS2814-RyR2 were normalised to RyR2, while pT286-CaMKII was normalised to CaMKII. Data are presented as Mean ± SEM. n = number of runs/differentiations. Normality of data was determined by Shapiro-Wilk test, while comparison was made using Mann Whitney's U-test. Ctrl, control; cTnC, cardiac troponin C; iPSC-CM, induced pluripotent stem cell-derived cardiac myocyte; ns, non-silencing; si, silencing; siRNA, small interfering RNA.

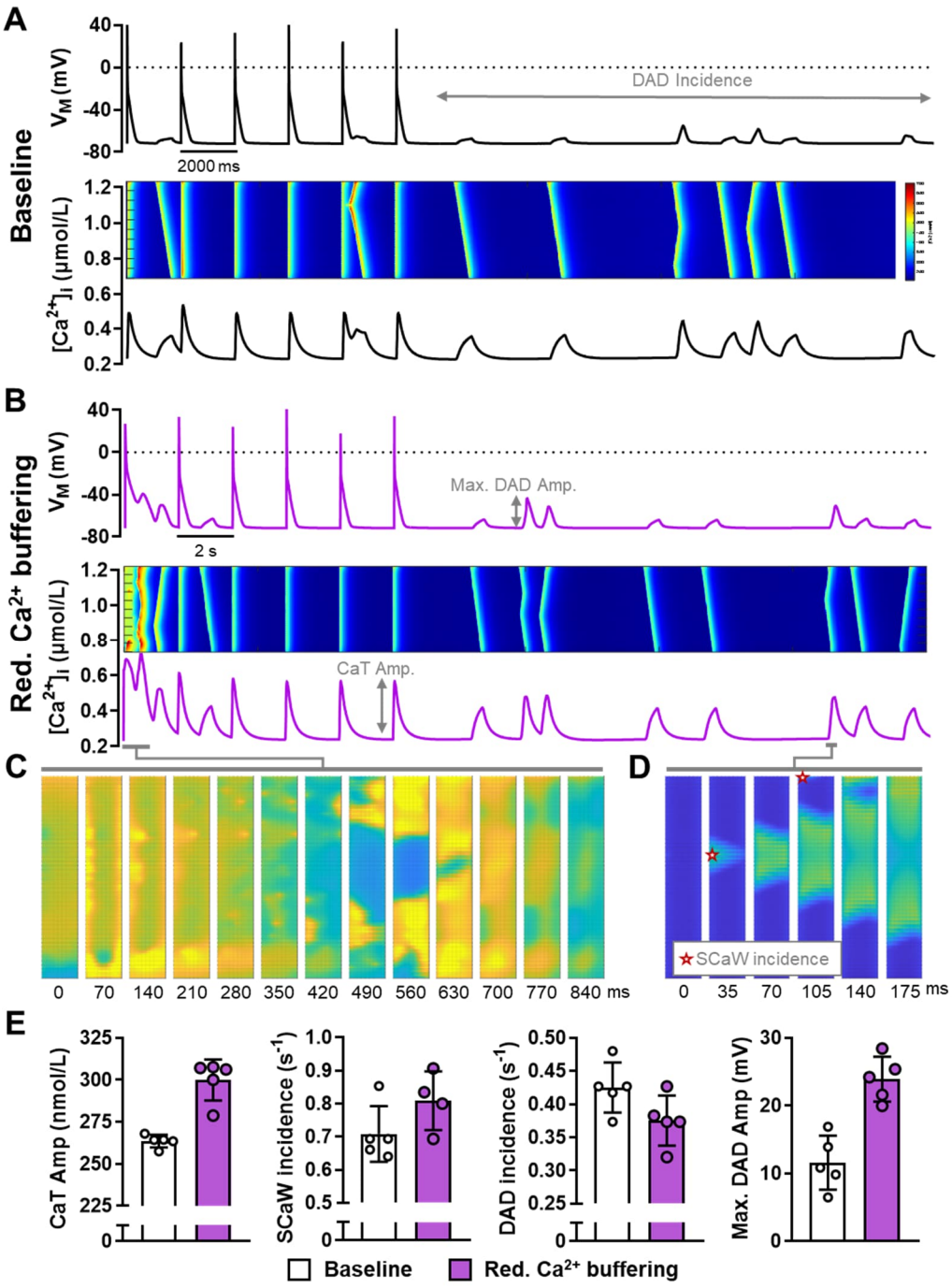

Figure S13. Computational analysis of the effect of reduced Ca^{2+} buffering on atrial Ca^{2+} handling. Simulated action potentials (Vm, top), longitudinal linescans (middle) and Ca^{2+} transients (CaT, bottom) occurring at 0.5 Hz, followed by a quiescent period showing SCaWs and DADs in a virtual atrial cardiac myocyte without (A, baseline) and with (B) reduced Ca^{2+} buffering. C, Two-dimensional snapshots showing local Ca^{2+} concentrations, highlighting prominent SCaWs upon an acute reduction in Ca^{2+} buffering and (D) the multiple simultaneous SCaWs underlying a DAD in a virtual atrial cardiomyocyte with reduced buffering. E, Data are presented as Mean ± SEM. n = number of simulations. DAD, Delayed afterdepolarization; SCaW, spontaneous calcium wave; Max., maximum; Red., reduced; Amp., amplitude.

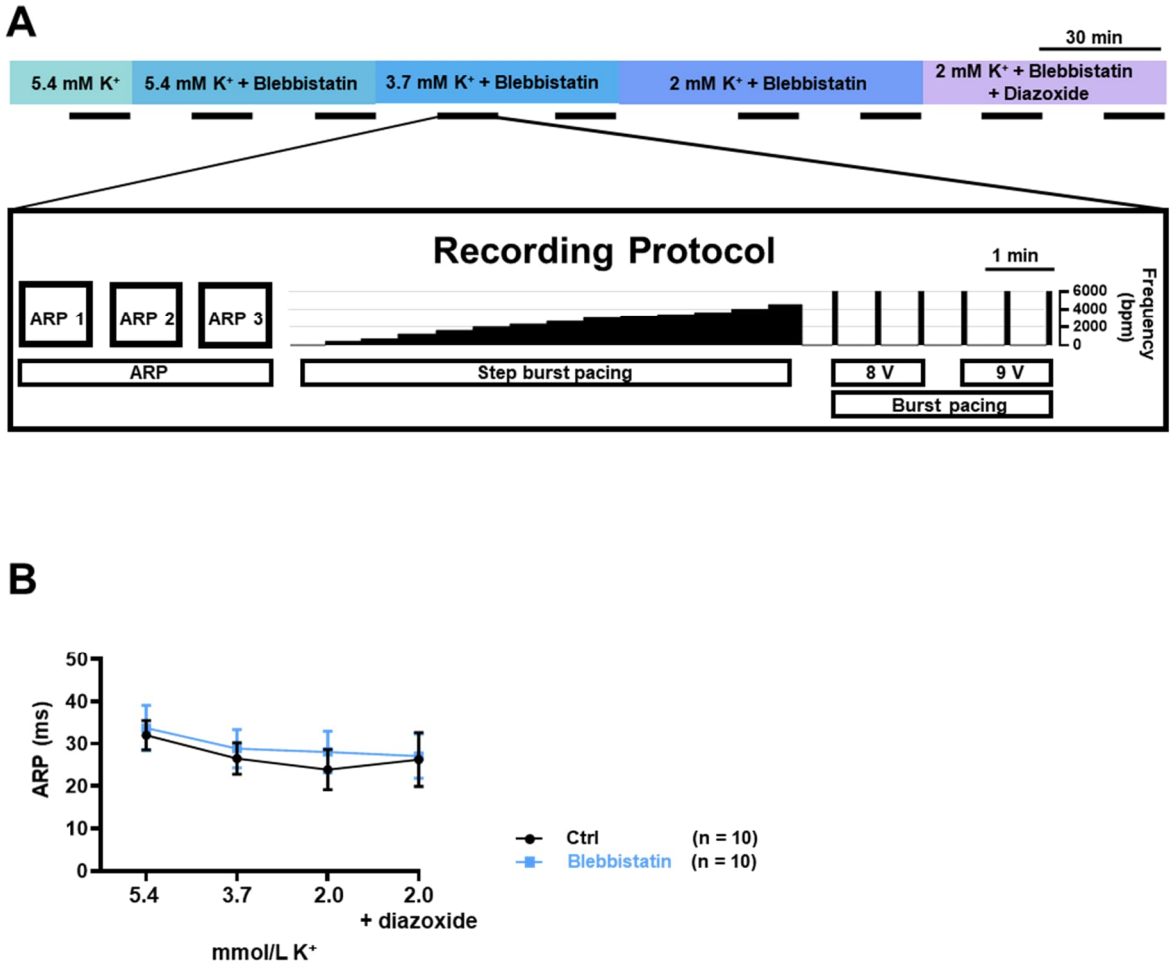

Figure S14. Summary of Langendorff experiments in mouse heart. A, Recording protocol, consisting of measurements of absolute refractory period (ARP) and application of burst pacing (control protocol omitted blebbistatin). B, Mean±SEM inducibility of atrial arrhythmic activity for each potassium level, differentiated by the presence or absence of blebbistatin. The graph highlights significant main effects of blebbistatin, $F(1, 70) = 4.26$, $P < 0.05$, and potassium, $F(3, 70) = 8.31$, $P < 0.0001$. Interaction $F(3, 70) = 0.41$, $P = 0.7439$. Comparison using two-way ANOVA followed by a Fisher's LSD post-hoc test. Ctrl, control.