SUPPLEMENTAL MATERIAL

Detailed Methods

Myocyte isolation. Myocytes from the left ventricle and both atria were isolated from male New Zealand White rabbits (37 animals, ~2.5 kg, Harlan Laboratories, Indianapolis, IN, USA). Rabbits were anaesthetized with IV injection of sodium pentobarbital (50 mg/kg) and heparin (1000 UI/kg). Hearts were excised, mounted on a Langendorff apparatus and retrogradely perfused via the aorta. After an initial 5 min washing step with oxygenated Ca^{2+}-free Tyrode solution (in mmol/L: 140 NaCl, 4 KCl, 10 D-Glucose, 5 Hepes, 1 $MgCl_2$, 10 BDM, 1000 UI/l Heparin; pH 7.4 with NaOH), the heart was perfused with minimal essential medium Eagle (MEM) solution containing 20 µmol/L Ca^{2+} and 22.5 µg/mL Liberase TH (Roche Diagnostic Corporation, Indianapolis, IN, USA) for 20 min at 37˚ C. The left ventricle and both atria were dissected from the heart and minced, filtered and washed in a MEM solution containing 50 µmol/L Ca^{2+} and 10 mg/mL bovine serum albumin. Isolated cells were washed and kept in MEM solution with 50 µM Ca^{2+} at room temperature (22-24˚C) and were used within 1-8 h after isolation. All procedures and protocols involving animals conform with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee.

Electrophysiological measurements: Electrophysiological signals were recorded from single cardiac myocytes in the whole-cell ruptured patch clamp configuration using an Axopatch 200A patch-clamp amplifier, the Axon Digidata 1440A interface and pCLAMP 10.2 software (Molecular Devices, Sunnyvale, CA). Current and AP recordings were low-pass filtered at 5 kHz and digitized at 10 kHz.

The standard external Tyrode solution was composed of (in mmol/L): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose; pH 7.4 with NaOH. All chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. For AP measurements and AP voltage clamp experiments patch pipettes (2–3 MΩ) were pulled from borosilicate glass capillaries (WPI, Sarasota, FL, USA) with a horizontal puller P-97 (Sutter Instruments, Novato, CA, USA) and filled with internal solution, containing (in mmol/L): 130 K^+ glutamate, 10 KCl, 10 NaCl, 0.33 $MgCl_2$, 4 MgATP, and 10 Hepes with pH adjusted to 7.2 with KOH. For simultaneous $[Ca^{2+}]_i$ measurements 75 µmol/L of Fluo-4 pentapotassium salt or 75 µmol/L Indo-1 pentapotassium salt (both from Molecular Probes/Life Technologies, Grand Island, NY) was added to the internal solution. Internal solutions were filtered through 0.22-µm pore filters. All experiments were performed at room temperature (22-24°C).

For AP measurements the whole-cell 'fast' current clamp mode of the Axopatch 200A was used and AP were evoked by 5 ms stimulation pulses of ~1.5-2 times higher

magnitude than AP activation threshold. Cell membrane potential (V_m) measurements were corrected for a junction potential of -10 mV.

For AP-clamp experiments voltage commands in form of atrial or ventricular AP_{CaT_Small} and AP_{CaT_Large} were derived as the average of a typical AP_{CaT_Small} and AP_{CaT_Large} recorded from three individual cells at a stimulation rate of 1.3 Hz and exhibiting CaT alternans with CaT_{Small}/CaT_{Large} ratio of ~0.4 (or AR=~0.6 as defined below). This resulted in two AP prototypes for each cell type that were used as command voltages, where AP_{CaT_Large} refers to the AP morphology recorded in connection with a large alternans Ca^{2+} transient, whereas AP_{CaT_Small} was recorded during a small alternans Ca^{2+} transient (cf. Fig. 1). Stimulation frequency was modified by proportionally changing AP duration and diastolic interval at rates >1.3 Hz, or by prolonging diastolic intervals between stimulations at frequencies <1.3 Hz. All AP-clamp experiments were conducted in standard Tyrode solution.

For I_{LCC} recordings all K^+ ions in intracellular and extracellular solutions were replaced with Cs^+ to eliminate K^+ currents. External solution was composed of (in mmol/L): 140 NaCl, 4 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose; pH 7.4 with NaOH. Pipettes were filled with internal solution, containing (in mmol/L): 130 Cs glutamate, 10 CsCl, 10 NaCl, 0.33 $MgCl_2$, 4 MgATP, and 10 Hepes with pH adjusted to 7.2 with CsOH. For I_{LCC} measurements cardiac myocytes were voltage clamped and held at -50 mV. I_{LCC} was triggered by depolarization steps to 0 mV lasting 100 ms. Peak I_{LCC} was measured as the difference between the inward peak current and the current at the end of the 100 ms depolarization pulse. For I_{LCC} I-V relationship myocytes were depolarized from a holding potential of −50 mV to test potentials between −50 and +50 mV with increments of 5 or 10 mV.

Cytosolic $[Ca^{2+}]_i$ measurements Simultaneously with electrophysiological recordings cytosolic $[Ca^{2+}]_i$ levels were monitored. For $[Ca^{2+}]_i$ measurements cells were loaded with fluorescent probes Fluo-4 pentapotassium salt or Indo-1 pentapotassium salt via the patch pipette. Fluo-4 fluorescence was excited with the 488 nm line of an argon ion laser and $[Ca^{2+}]_i$-dependent Fluo-4 signals were collected at 515 nm using a photomultiplier tube. Background-subtracted fluorescence emission signals (F) were normalized to resting fluorescence (F_0) recorded under steady-state conditions at the beginning of experiment, and changes of $[Ca^{2+}]_i$ are presented as changes of F/F_0. Indo-1 fluorescence was excited at 357 nm (Xe arc lamp) and emitted cellular fluorescence was recorded simultaneously at 410 nm (F_{410}) and 485 nm (F_{485}) with photomultiplier tubes. F_{410} and F_{485} signals were background subtracted and changes of $[Ca^{2+}]_i$ were expressed as changes in the ratio $R=F_{410}/F_{485}$. Measurements, data recording and digitization were achieved using the Axon Digidata 1440A interface and pCLAMP 10.2 software.

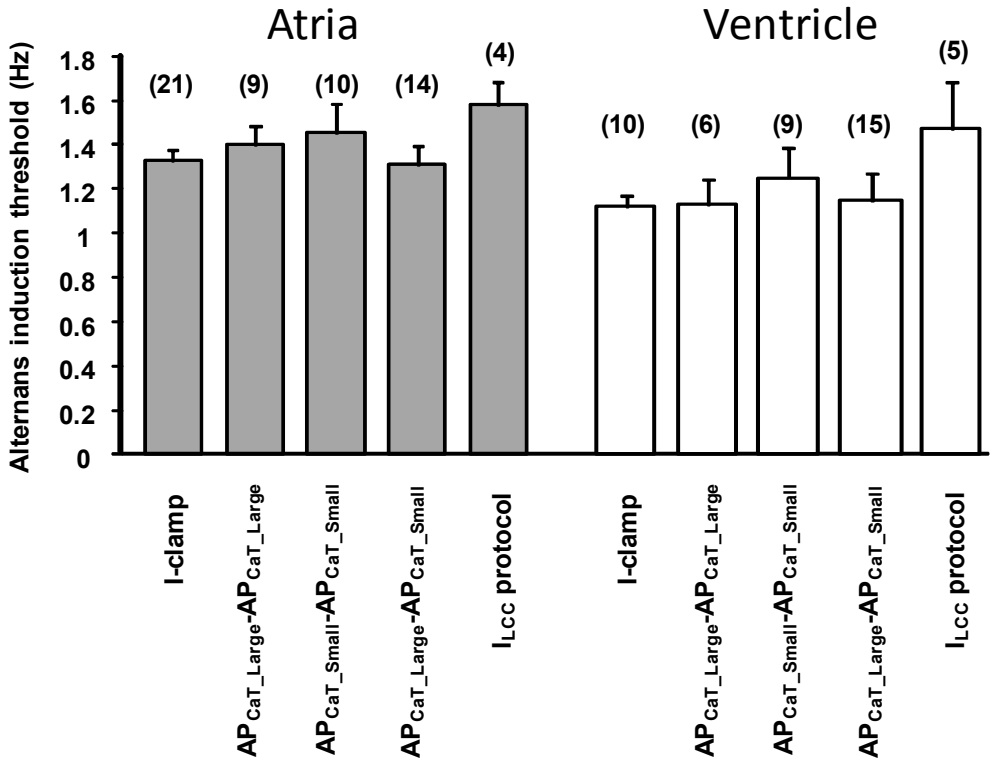
CaT alternans was induced by incrementally increasing the pacing frequency (frequencies used: 0.5, 0.8, 1.0, 1.3, 1.6, 1.8, 2.0 Hz) until stable alternans was observed. The average frequencies at which stable CaT alternans was observed in current- and voltage-clamp experiments in atrial and ventricular myocytes are summarized in Supplemental Figure I. The degree of CaT alternans was quantified as the alternans ratio (AR). AR=1-CaT_{Small}/CaT_{Large}, where CaT_{Small} and CaT_{Large} are the small- and large-amplitude CaTs from a pair of alternating CaTs. CaTs were considered alternating when the beat-to-beat variation in CaT amplitude exceeded 10% (AR of >0.1)[1]. The amplitude of a CaT was measured as the difference in R or F/F_0 measured immediately before the stimulation pulse and the peak of the CaT.

Data analysis and presentation. Results are presented as individual observations or as means ± SEM. Statistical significance was evaluated using Student's *t*-test. Unless stated otherwise, *n* represents the number of individual cells and differences were considered significant at $p<0.05$.

References:

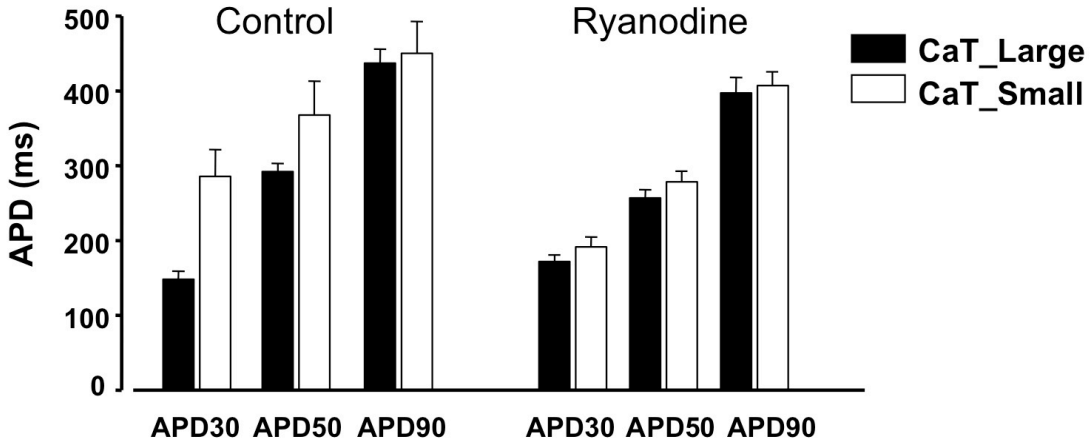
1. Kockskamper J, Zima AV, Blatter LA. Modulation of sarcoplasmic reticulum Ca^{2+} release by glycolysis in cat atrial myocytes. *J Physiol*. 2005;564:697-714

Supplemental Figures



Supplemental Fig. I. Alternans induction threshold for different current- and voltage-clamp protocols.

Alternans induction threshold was determined as the mean of the lowest pacing frequencies at which CaT alternans were observed with different pacing modes used. There were no significant differences between thresholds in current-clamp, same-shape AP-clamp or alternating AP-clamp experiments in both atrial and ventricular cells. However, a tendency to higher pacing rates to elicit CaT alternans was observed in voltage-clamp experiments using step-like depolarizations (I_{LCC} protocol). Also, ventricular cells tended to exhibit lower alternans induction thresholds than atrial myocytes.



Supplemental Fig. II. Application of ryanodine suppresses beat-to-beat alternation in APD.

Summary data for APD30, APD50 and APD90 before (control) and after application of 10 µmol/L ryanodine in atrial myocytes (n=4).