Supplemental Material

Myocyte isolation and culture

Myocytes were isolated by enzymatic digestion\(^1\) and kept in a HEPES buffered solution at room temperature (20-24 °C), until use. Unless otherwise specified, experiments were performed at room temperature. For culture, isolated cells were resuspended in DMEM medium containing (in g/l) 0.017 ascorbic acid, 0.4 L-carnitine, 0.66 creatine, 0.62 taurine, 50U/ml penicillin, and 50 U/ml streptomycin, and counted. Myocytes were plated at a density of \(~2 \times 10^4\) rod-shaped cells/ml into culture dishes for 1h to allow cell attachment. After this period, the culture media was changed for a fresh one and infected with viral particles according to the experiments performed (see results). After 24 h of culture, the cells were photographed to assess transfection efficiency and collected to perform functional experiments.

Indo-1 fluorescence and cell shortening measurements

Cells were placed on the stage of an inverted microscope (Nikon Diaphot 200) adapted for epifluorescence, continuously superfused with HEPES buffered solution (pH 7.4) at a constant flow of 1 ml/min and field stimulated via two platinum electrodes on either side of the bath, at 0.5 Hz. The ratio of the Indo-1 emission (410 and 490 nm) was taken as an index of \(\text{Ca}^{2+}_i\). The SR \(\text{Ca}^{2+}\) content was evaluated by the amplitude of the \(\text{Ca}_iT\) induced by 15 mM caffeine. Resting cell length and cell shortening were measured by a video-based motion detector (Crescent electronics, UT, USA) and stored by software for an off-line analysis (PowerLab/400 ADInstruments).

Confocal imaging of intact cardiac myocytes
Confocal images of Ca\(^{2+}\) sparks were taken in the line scan mode\(^2\). Cells were exited with the 488nm line of an argon laser and fluorescence was collected at >515nm. Each image consisted of 512 line scans obtained at 4 ms intervals. Data were visualized using Leica Application Suite and Ca\(^{2+}\) sparks were measured using the “Sparkmaster” plugin for ImageJ\(^3\). Sparks were obtained in quiescent cells after 20 min stimulation at 0.5 Hz either in the presence or absence of ouabain 50 µmol/l.

**Western Blot**

Protein was measured by the Bradford method using BSA as standard. Lysates (~90 µg of total protein per gel line) were seeded in a 10% SDS polyacrylamide gel\(^4\) and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with the antibodies raised against phospholamban (PLN) (ABR, California, USA) Phospho-Thr17-PLN (Badrilla, Leeds, UK) CaMKII (Chemicon International, Lake Placid, NY) phospho-Thr286-CaMKII, (Badrilla, Leeds, UK) phospho-Ser2814-RyR (Badrilla, Leeds, UK) and Anti GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA) for normalization. Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Amersham Biosciences) using a Chemidoc Imaging System. The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH).

**Adenoviral Infection**

Plated rat myocytes were infected with adenoviruses at a multiplicity of infection (MOI) of 100 and cultured for 24 hrs. The verification of the transgene expression was monitored by western blot and GFP fluorescence at an excitation wavelength of 480 nm after the culture period\(^1\).
In vivo ECG measurements

ECGs were acquired at 40 KHz sampling rate. Recordings were performed after 20 min intraperitoneal injection of 10mg/kg ouabain and for a period of 60 min. Arrhythmias were assessed by quantifying the incidence of ventricular ectopic beats (premature or ventricular escape beats) and sustained ventricular tachycardia during the 60 min recording period. Similar experiments were conducted in mice pretreated with the CaMKII inhibitor KN 93 (30 µmol/Kg IP). This dose of KN 93 has been shown to prevent arrhythmic processes of different etiology\(^5,6\).

To avoid restraining mice during the 60 minutes ECG recording period a more physiologic ECG recording system in which the mice can move freely around the cage was designed and used in a group of mice. As shown in the photograph, we made a cloth vest holding the ECG electrodes connected through very thin cables to the acquisition system. This vest was placed on the mouse’s chest and ECG was recorded during one hour in the absence and presence of ouabain and ouabain + KN93. Disconnected vests were placed on the mice for 24 hrs prior to the experiment for their habituation. Using this more physiological approach, we obtained similar results to those using the retrained animal.
Ouabain Doses

The therapeutic and toxic levels of both ouabain and digoxin are more than 100 times higher in rodents than in humans. The dose of ouabain used in this study (50 µmol/l) has been previously shown to be arrhythmogenic. This dose is in the lower limit of toxicity in the rat heart, a species with extremely low sensitivity to ouabain. We chose this dose of ouabain because in control experiments (not shown) we observed that it was able to systematically and reproducibly produce arrhythmias and utilized this as a model to study the underlying subcellular mechanisms involved in ouabain-induced arrhythmias.

The dose of ouabain used in our in vivo experiments was 10 mg/Kg I.P. In control experiments we observed that ouabain at an order of magnitude below the dose used, did not promote arrhythmias within the first hour following administration, whereas ouabain at an order of magnitude above the one used, killed the animals during the first hour. Furthermore, the dose of ouabain recognized as the i.p. lethal dose 50 (LD50) in the mouse is 11 mg/kg. Thus, for our in vivo ECG measurements we used a similar dose (10 mg/Kg).

Data Analysis:

Data sets resulting from the quantification of random events (NSE, Ca2+ spark characteristics and wave frequency), were summarized in box and whisker plots. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbol in the box denotes the mean of the data. Non parametric statistical tests were used to assessed whether differences between groups were statistical significant. Kruskal Wallis one-way ANOVA analysis of variance followed by Dunn’s method was used
for multiple comparisons and Mann-Whitney Rank sum test was used for two sample groups.

References


4. Mundina-Weilenmann C, Vittone L, Ortale M, de Cingolani GC, Mattiazi A. Immunodetection of phosphorylation sites gives new insights into the


