Online Supplementary Data

Materials and methods

Isolation of Mouse SAN

To isolate SAN, mice are euthanized with sodium pentobarbital and the heart is quickly excised into Tyrode solution (36 ± 0.5 ºC) consisting of (in mM) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.55 glucose, and 5 HEPES, with pH adjusted to 7.4 with NaOH.

Isolation of Single Mouse SANC

A strip of tissue containing the SAN region, measuring ~0.5 mm × ~1 mm, is restricted by anatomic landmarks and dissected out as described previously [9, 10]. The SAN strips are cut into 3-5 smaller strips and rinsed in a 'low Ca^{2+}, low Mg^{2+}' solution containing (in mM) 140 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 0.2 $CaCl_2$, 50 taurine, 18.5 glucose, 5 HEPES and 1 mg/ml bovine serum albumin (BSA), with pH adjusted to 6.9 with NaOH. The rinsed SAN tissue strips are digested in the same 'low Ca^{2+}, low Mg^{2+}' solution containing collagenase (229 u/ml, type II, Worthington Biochemical Corporation), elastase (1.9 u/ml, Sigma) and protease (0.9 u/ml, type XIV, Sigma) for 15 to 25 min. The tissue is then transferred to KB solution which contains (in mM), 100 potassium glutamate, 10 potassium aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose and 5 HEPES, with pH adjusted to 7.4 with KOH). Single SANC are released by mechanically agitating the digested tissue strip, by gentle pipetting of tissue strips. SANC are identified under light microscopy by their spindle shape and small size with centered single nuclei (e.g., Figures 2, 4).

List of antibodies used in this study

The following antibodies are used: anti-HCN4 (hyperpolarization-activated cyclic nucleotide-gated channel 4, 1:100, Alomone, Israel); anti total phospholamban (PLB, 1:200, Badrilla, Leeds, UK), anti-phosphorylated phospholamban (SER16) (PLB-PS16, 1:200, Badrilla, Leeds, UK); anti-NCX1 (1:200, clone R3F1; Swant. Bellinzona, Switzerland); anti-RyR2 antibody (1:200, clone C3-33; Affinity BioReagents, Golden, CO), anti-SERCA2 antibodies (1:500, clone IID8 and Clone 2A7-A1; Affinity BioReagents, Golden, CO), anti-Caveolin-3 (Cav-3, 1:200, BD Biosciences, NJ), anti-Calsequestrin (CSQ, 1:200, Cat#: ab3516, Abcam, MA). The specificity of each antibody has been tested elsewhere [17]. Two secondary antibodies, Alexa Fluor 488 donkey anti-rabbit IgG and Rhodamine red donkey anti-mouse IgG (1:200 dilution; Molecular Probes) are employed to detect various primary antibodies.

Immunolabeling of SAN tissue section and single SANC

Immunocytochemistry was carried out on isolated cells and tissue sections. Cells were plated onto laminin-treated slides and allowed to settle for 30 min. Tissue sections were cut perpendicular to the Crista Terminalis using a cryostat. Cells and sections were fixed in 4 % paraformaldehyde (Sigma) for 10 min, washed three times with phosphate buffer solution (PBS) over 30 min, permeabilized by 0.1% Triton X-100 (Sigma) for 30 min, washed three times with PBS over 30 min, and blocked with 1% bovine serum albumin (BSA; Sigma) and 4% normal Goat serum (invitrogen, USA) in PBS for 1–2 h before application of primary antibody. Primary antibodies were diluted in 1% BSA and 4% normal goat serum in PBS. Cells and sections were incubated with primary antibody (at an appropriate concentration) at 4°C overnight, briefly washed in PBS and then Alexa Fluo-488 goat anti-rabbit or Alexa Fluo-561

goat anti-mouse secondary antibodies (1: 200 dilution, Invitrogen, USA) were applied. Cells and sections were washed three times with PBS and then mounted with Vectashield (H-1000; Vector Laboratories, USA) and coverslips were sealed with nail polish. In control experiments on isolated cells and tissue sections, no primary antibody was used and no labelling was detected.

Confocal imaging of local subsarcolemmal Ca^{2+} releases in single permeabilized SANC

Intact mouse SANC are plated on laminin-coated (20 µg/ml) in 35 mm #zero glass bottom petri dishes (MatTek Cultureware) for 20 minute to attach. After superfusion with normal Tyrode solution (with 1.8 mM Ca^{2+}), only spontaneously beating SANC are selected and then permeabilized with saponin (0.01 % for 2 min) in an "internal" solution containing (in mM): K aspartate, 100; KCl, 25; NaCl, 10; MgATP, 3; $MgCl_2$, 0.81 (free [Mg^{2+}] ~1 mM); HEPES, 20; EGTA, 0.5; phosphocreatine, 10 and creatine phosphokinase, 5U/ml; pH 7·2. After carefully washing out saponin, the permeabilized solution is changed to a continuous superfusion with a recording experimental solution, as above, but with added 30 µM fluo-4 pentapotassium salt (Molecular Probes, Eugene, OR) and 100 nM free [Ca^{2+}]. The total Ca^{2+} concentration required to generate a free [Ca^{2+}] is calculated using a computer program (WinMAXC 2.50, Stanford University, CA). The temperature is maintained at 35±0.5 ^{o}C by the lens heater and pre-heater (TC2BIP 2/3Ch Bipolar Temp controller from Cell MicroControls).

Ca^{2+} signals recording in skinned cells

Spontaneous local Ca^{2+} releases (LCR) in skinned cells are recorded as previously described.[1] Briefly, all Ca^{2+} signals are recorded in skinned cells loaded with the Ca^{2+} indicator, Fluo-4 salt, by line-scanning confocal microscopy (Carl Zeiss, with a

ZEISS Plan-NEOFLUAR 63x/1.40 oil objective, Inc., Germany) an argon laser at 488-nm excited and the fluorescence signal is collected at wavelengths of >515 nm in line scan mode oriented along the cell periphery.

Image analysis

Ca^{2+} images are analyzed as previously described. [1,2] Briefly, Fluo-4 salt of Ca^{2+} signals were presented as F/F_0 (normalized Ca^{2+} signal) or as a absolute free Ca^{2+} concentration ($[Ca^{2+}]_i$) change (ΔCa^{2+}, nmol/L). $[Ca^{2+}]_i$ values in cells are evaluated from respective F/F_0 values using a previously described formulation:[2] $[Ca^{2+}]_i=K_d\cdot(F/F_0)/(K_d/[Ca^{2+}]_r+1-F/F_0)$, where $[Ca^{2+}]_r$ is $[Ca^{2+}]_i$ at rest (no Ca^{2+} releases) that was set to 100 nM in our permeabilized cells. K_d (1 μmol/L) is the dissociation constant for Ca^{2+}-bound fluo-4. Images are analyzed by custom-made (Victor A. Maltsev) imaging software based on Delphi-7 developing software and with IDL software (5.4, Research Systems, Boulder, CO). Power spectra and autocorrelation functions of Ca^{2+} signals are calculated by a Clamp Fit program (version 9.2). LCR spatial size was indexed as the full width at the half maximum amplitude (FWHM), and its duration characterized as the full duration at half maximum amplitude (FDHM). The individual LCR signal mass (SM) was measured as follows: SM= FWHMXFDHMX1/2$\Delta F/F_0$ (where $\Delta F/F_0=F/F_0-1$). The total integrated LCR signal mass is calculated as the sum of signal masses of individual LCRs. For the IBMX study, a crude index of the relative numbers of activated RyRs within an LCR for a given cell is calculated as total integrated LCR signal mass divided on the smallest individual LCR signal mass (SM) before the drug application. In addition LCR number and total integrated LCR signal mass of each cell are normalized per 100 μm of the linescan image size during a one-second time interval.

Drugs

The following drugs are applied in this study: Isoproterenol (ISO, 1 µM, cat#420355, Calbiochem), atropine (10µM cat#A0132, Sigma Aldrich), propranolol (2.5 µM, cat# P0884 Sigma Aldrich), a specific protein kinase A inhibitor 14-22 Amide, myristoylated (PKI, 15 µM, cat#4476485, Calbiochem (skinned cell experiments))_, variable concentrations of H89 (N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl, cat#371963, Calbiochem), Cyclopiazonic acid (CPA, 5 µM, cat#C1530, Sigma), 3'-isobutylmethylxanthine (IBMX, 100 µM, 5 µM(for skinned cell experiments) cat#15879, Sigma), MDL, an AC inhibitor 12330A (100 µM, cat# M128, Sigma), various concentrations of ryanodine (cat#559276, Calbiochem, CA USA), and 20 mM Caffeine (cat#C0750, Sigma), and EGTA (10 mM, 0.5 mM cat#E0396, Sigma)

ON-LINE SUPPLEMENT Figures

Online Supplement Figure 1: Voltage- and time-dependent gating properties of an ensemble of mutually interacting surface membrane ion channels governs the rate and rhythm of spontaneous action potential (AP) firing of the heart's pacemaker cells by controlling spontaneous membrane depolarization between APs. Because this ensemble of electrogenic membrane proteins can, *in silico,* generate rhythmic APs, it can be envisioned as a surface membrane "clock" (M clock). In the context of the APs that it produces, the M clock regulates cell Ca^{2+} balance in a given steady state via Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} efflux through the Na^{+}/ Ca^{2+} exchanger (NCX). The electrogenic and regulatory molecules on the surface membrane of sinoatrial nodal cells are strongly modulated by Ca^{2+} and phosphorylation. Phosphorylation-dependent intracellular Ca^{2+} cycling is also an integral player in controlling pacemaker cell automaticity.2 Spontaneous, localized, and critically timed local subsarcolemmal Ca^{2+} releases generated by the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) occur during late diastolic depolarization. The SR has been referred to as an intracellular "Ca^{2+} clock" because the submembrane Ca^{2+} oscillations that it generates are periodic, occur when surface membrane function is experimentally eliminated, and also exist *in silico.* However, in nature, neither the M clock nor the Ca^{2+} clock exists in isolation of each other. Ca^{2+} and phosphorylation-dependent coupling of the functions of molecules composing the

subsystem M and Ca^{2+}clocks creates a complex, robust, coupled-clock pacemaker system that ensures stable rhythmic firing of cardiac pacemaker cells.

Online Supplement Figure 2: Ca^{2+} cycling proteins are expressed in SAN regions. SAN center, peripheral and atrial myocytes in CT are immunolabeled with both HCN4 and RyR2, NCX1 or SERCA2 antibody. In all three regions, RyR2, NCX1 and SERCA2 are abundantly expressed.

Online Supplement Figure 3: Single isolated ventricular myocyte and SANC are labeled with CSQ antibody. Two different patterns of sub-cellular distribution are found in these two types of cells. CSQ shows uniform distribution throughout the ventricular myocyte, while a dominant sub-membrane distribution pattern is seen in SANC. Scale = 20 μM.

Online Supplement Figure 4: A. Average dose-response of spontaneous SANC beating rate to ryanodine or H89. B. Representative examples of the effects of H89 or MDL on SANC beating rate.

Online Supplement Figure 5: Confocal image of a single SANC double labeled with total PLB (red) and PS-16 PLB (green). Note the PS-16 PLN shows a sub-sarcolemmal enriched pattern (white arrows) in these cells. This enriched pattern is further revealed in the pseudo-color image in the right panels.

Online Supplement Figure 6: Confocal images of isolated ventricular myocytes immuno-labeled with PLB-PS16 (green) and PLN total (red) in (A) untreated (basal), β-adrenergic stimulation (ISO, 1 μM), PKA inhibition (PKI, 15 μM or H89?CONC), (B) PDE inhibition (IBMX, 100 μM) and (C) AC inhibition (MDL, 100 μM). Scale = 20 μm.

Online Supplement Figure 7: The effect of Isoproterenol to augment the SANC beating rate is blunted in the presence of CPA or ryanodine.

Online Supplement References

1. Bogdanov KY, Vinogradova TM, Lakatta EG. Sinoatrial nodal cell ryanodine receptor and Na+-Ca2+ exchanger: molecular partners in pacemaker regulation. Circ Res 2001;88:1254-8.

2. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. Science 1993;262:740-4.