SUPPLEMENTAL MATERIAL

A genetic model of cardiac CaMKII inhibition

Transgenic AC3-I and AC3-C mice were made in our laboratory.[1] The transgenic mice have cardiomyocyte over-expression of a CaMKII inhibitory peptide (AC3-I) or an inactive scrambled control peptide (AC3-C) under control of the cardiac-specific α myosin heavy chain promoter. The α myosin heavy chain promoter drives expression of genes in cardiac myocytes and SANC,[4] so AC3-I and AC3-C are expressed in SANC. Both AC3-I and AC3-C were fused to enhanced green fluorescent protein (eGFP) to mark the cellular and tissue distribution of the transgenically expressed protein.

Langendorff perfused hearts

ECG recording from Langendorff perfused hearts was performed as described previously.[2] Excised hearts were quickly mounted on a modified Langendorff apparatus (HSE-HA perfusion systems, Harvard Apparatus) for retrograde aortic perfusion at a constant pressure of 80 mm Hg with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution consisting of (mM) 25 $NaHCO_3$, 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 0.5 Na-EDTA, and 15 glucose, with pH equilibrated to 7.4. The perfused heart was immersed in the water-jacked bath and was maintained at 36 °C. The ECG was continuously recorded with Ag^+-AgCl electrodes, which were positioned around the heart in an approximate Einthoven configuration. The hearts were perfused with different concentration of BayK or isoproterenol after equilibration for 25 mins. Each dose of chemicals was perfused for at least 5 mins.

SAN cell (SANC) isolation

Single SANCs of mouse and dog were isolated as described in detail, [3-4] and the protocol was approved by the Animal Care and Use Review Board at The University of Iowa and conformed to all regulations for animal use including guidelines of American Physiology Society. Briefly, the heart was quickly excised and put into cold Tyrode's solution. The solution consists of (mM) 140 NaCl, 5.0 HEPES, 5.5 glucose, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, pH adjusted to 7.4 with NaOH. The SAN region, delimited by the crista terminalis, atrial septum and orifice of superior vena cava, was dissected from heart. The SAN tissue was then chopped into small chunks, and was incubated with a 'low Ca^{2+}' solution twice at 37 ^oC, each for 5 mins. This solution contains (mM) 140 NaCl, 5.0 HEPES, 5.5 glucose, 5.4 KCl, 0.2 $CaCl_2$, 0.5 $MgCl_2$, 1.2 KH_2PO_4, 50 taurine and 1 mg/ml bovine serum albumin (BSA), with pH adjusted to 6.9 with NaOH. The SAN tissue was then digested in 10 ml of enzyme solution with collagenase type I (Worthington, Lakewood, NJ), elastase (Worthington, Lakewood, NJ), and protease type XIV (Sigma, St Louis, MO) in 'low Ca^{2+}' solution for about 30 min. The tissue was then transferred to 10 ml of Kraft-Brule. The tissue was then gently agitated using a glass pipette for 5 mins. The dissociated cells were then stored at 4 ^oC and were used within 8 hours after isolation.

Electrophysiology

The cell suspension was placed in a chamber mounted on an inverted microscope and was allowed to settle for at least 5 mins. The perfusion time for BayK was at least 5 mins and 20 mins for ryanodine. SANCs were identified by their characteristic morphology (spindle or spider shape) and spontaneous activity.

Spontaneous APs were recorded using the perforated patch-clamp technique in Tyrode's solution at 36 ± 0.5 ^{o}C with the pipette filled with (mM) 30 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 $CaCl_2$, 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, with pH adjusted to 7.2 with KOH. Amphotericin B (240 μg/ml) was added into the pipette solution to form the perforated patch.

I_f was recorded with peforated patch.[3] patch pipettes had a resistance of 2-4 MΩ when filled with intracellular solution, containing (mM): 120 potassium aspartate, 25 KCl, 4.0 $MgCl_2$, 10 EGTA, 4.0 KATP-, 2.0 NaGTP, 2.0 phosphocreatine, 5.0 HEPES, 1.0 $CaCl_2$, and the pH was adjusted to 7.2 with KOH, The extracellular solution contained (mM): 130 NaCl, 5.0 KCl, 2.0 $MgCl_2$, 1.8 $CaCl_2$, 5.0 HEPES, the pH was adjusted to 7.4 with NaOH. After the initial current recordings, 0.5 mM $BaCl_2$ was applied to extract Ba^{2+}-sensitive K^+ component. The voltage steps were applied for 1.0 s ranging from −120 mV to −40 mV in 10 mV increments at the holding potential of -35 mV.

I_{Ca} was measured with conventional whole mode. Depolarizing voltage pulses (300 ms in duration) to various potentials (from –50 mV to +50 mV in 10 mV step) were applied from a holding potential of –60 mV. The pipette (intracellular) solution comprised (mM): 120 CsCl, 10 EGTA, 10 HEPES, 10 tetraethylammonium chloride (TEA), 5.0 phosphocreatine, 3.0 $CaCl_2$, 1.0 MgATP, 1.0 NaGTP, the pH was adjusted to 7.2 with 1.0 N CsOH. The bath (extracellular) solution comprised (mM): 130 TEA, 25 HEPES, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 4-aminopyridine (4-AP), pH was adjusted to 7.4 with TEAOH.

I_{NCX} was recorded as described previously.[5] The inward currents were induced with a voltage ramp protocol from holding potential of –60 to –45 mV.

Immunofluorescence studies

Isolated SANCs were gently washed with phosphate-buffered saline (PBS, pH 7.4) and immediately fixed with 2% paraformaldehyde for 20 min. Cells were blocked/permeabilized in PBS containing 0.075% triton X-100, 2 mg/ml BSA and 3% fish gelatin, and incubated in primary antibody against autophosphorylated CaMKII (pCaMKII) (Genescript, NJ) overnight at 4 °C. Following PBS washes for three times, cells were incubated in secondary antibody (Molecular Probes, CA) for 2 hours at room temperature and mounted with Vectashield (Vector, CA) and #1 coverslips. Images were collected on Zeiss 510 Meta Confocal microscope (63 power oil 1.40 NA (Zeiss, Germany), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software.

Intracellular Ca^{2+} transients and sparks

Intracellular Ca^{2+} transients and sparks were observed using the confocal microscope as described previously.[3] Briefly, SAN cells were loaded with 1 μM rhode-2 acetoxymethyl ester for 10 min, and then rested for 20 min for deesterification in normal Tyrode's solution. The cells were then placed on the recording chamber mounted on a confocal Ca^{2+} imaging system (LSM510, Carl Zeiss MicroImaging), and

were perfused with either normal Tyrode's solution or various drugs at 36 ± 1 °C (Temperature Controller, TC2BIP, Cell MicroControls). Line scan mode was used at a speed of 1.92 ms per line and the scan lines were applied along the cell edge, because Ca^{2+} release occurs mostly in the SANC periphery. Ca^{2+} transients and sparks were analyzed offline with IDL program (Research System Inc.). Only Ca^{2+} sparks occurring within the late one third of diastole were used for analysis, as our previous studies with simultaneous recordings of AP and Ca^{2+} images showed that those areas corresponded to the AP phase 4 DD in SANCs.

Western blot

Western analyses were performed to measure the phosphorylated (at threonine17 and serine16) and total phospholamban expression in SAN tissue isolated from WT, AC3-C and AC3-I mice. SAN extracts were prepared in an extraction buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 1% (vol/vol) Nonidet P-40; 0.5% sodium deoxycholate; and 5 mM EDTA) with protease and phosphatase inhibitors. Protein extracts (10 μg total protein/ lane) were fractionated on bis-Tris NuPAGE gels (Invitrogen) with 1x MES-SDS running buffer and transferred to PVDF membrane (Bio-Rad). After blocking the nonspecific binding by 10% nonfat powdered milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.01% Tween 20), membranes were incubated with antibodies against phosphorylated-Thr17 (pT17) or phophorylated Ser-16 (pS16) (Cyclacell, U.K.). After washing, the membranes were incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham). Signals from bound antibodies were visualized by using the enhanced chemiluminescence detection system (Roche). Blots were then stripped and probed again for phospholamban using mouse monoclonal antibody (GE healthcare, U.K.) as described above except an HRP-conjugated anti-mouse secondary antibody was used. Protein bands were quantified using Quantity One (BioRad) from scanned autoradiograms.

Drugs

Ryanodine was purchased from Alexis Biochemicals (Plymouth Meeting, PA). All other chemicals were purchased from Sigma (St Louis, MI).

--Fig S1--

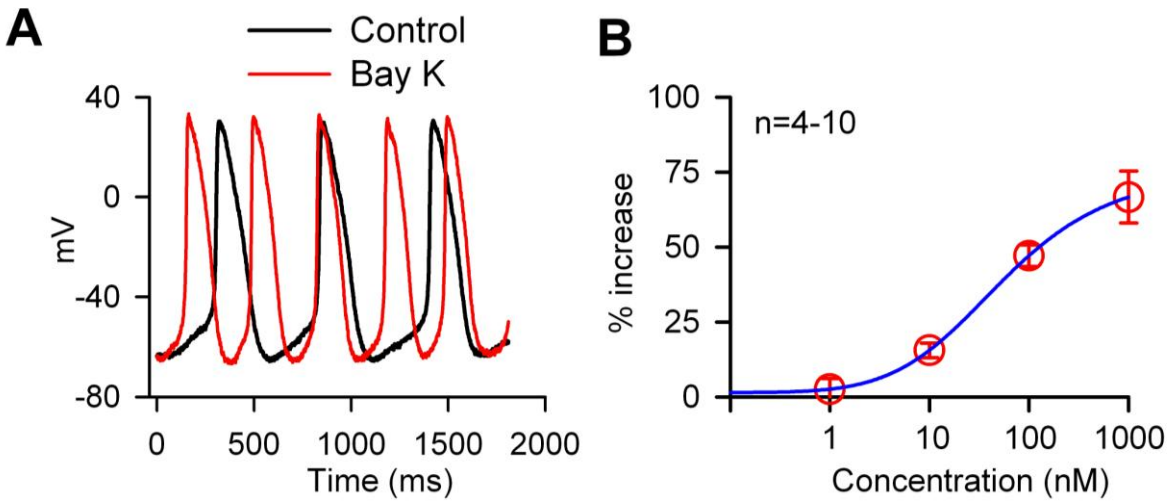

Fig S1 BayK increased the pacemaking activity of dog SANCs independent to β-AR stimulation. **A.** Representative AP traces recorded from a dog SANC before (black line) and after 1 μM BayK (red line). **B. Boltzmann fit** dose-response relationship of pacemaking activity with BayK in isolated dog SANCs, n=4-10 for each dose.

--Fig S2--

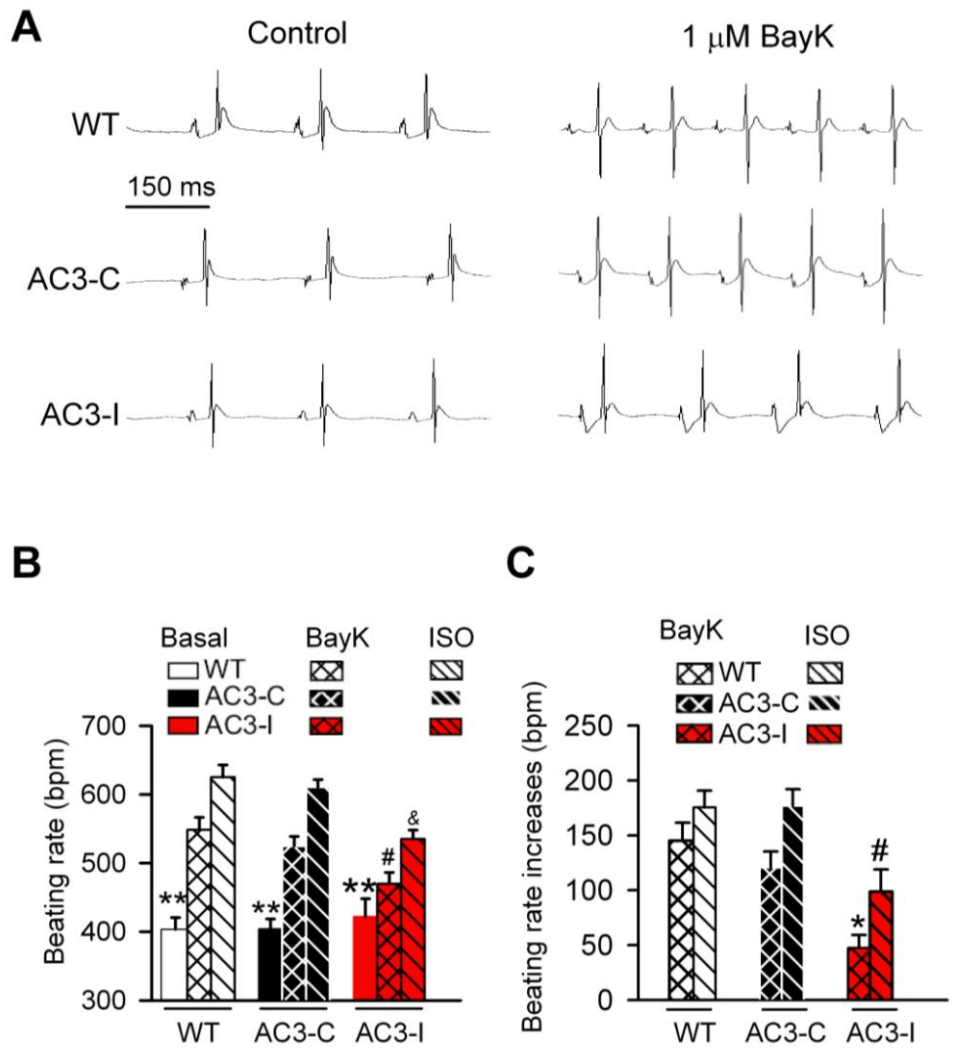

Fig S2 Langendorff perfused hearts showed that heart rate increase by 1 μM BayK in AC3-I mice was blunted compared to WT and AC3-C mice. **A.** Representative ECG traces of Langendorff perfused hearts recorded before and after BayK from the three genotype mice. **B.** Heart rates under control conditions, in the presence of BayK and isoproterenol from the three genotype mice. n≥5 for each groups **P<0.01, control vs BayK or isoproterenol; # P<0.05 AC3-I with BayK vs WT or AC3-C with BayK. & P<0.05 AC3-I with isoproterenol vs WT or AC3-C with isoproterenol. **C.** Rate increases by BayK in AC3-I mice were smaller than those in WT and AC3-C mice. n≥5 for each genotype, *P<0.05, AC3-I with BayK vs WT or AC3-I with BayK. # P<0.05, AC3-I with isoproterenol vs WT or AC3-I with isoproterenol.

--Fig S3--

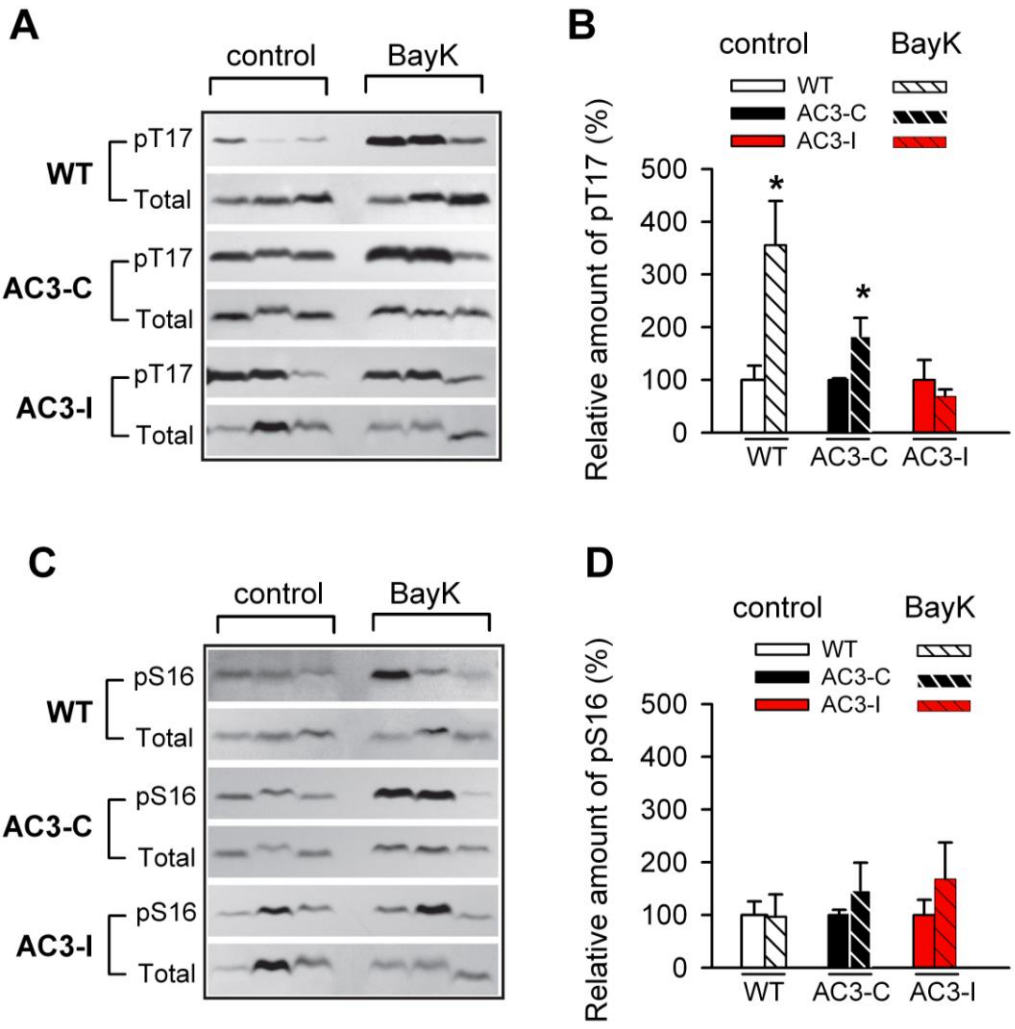

Fig S3 BayK increased CaMKII-dependent phosphorylation of phospholamban at threonine 17 (pT17), but did not significantly alter PKA-dependent phosphorylation of Phospholamban at serine 16 (pS16) in SAN tissue. **A.** Representative blots of pT17 and total phospholamban from WT, AC3-C and AC3-I mice. **B.** Relative amount of pT17 in control and at the presence of 1 μM BayK from the three genotypes. **C.** Representative blots of pS16 and total phospholamban from WT, AC3-C and AC3-I mice. **D.** Relative amount of pS16 in control and at the presence of 1 μM BayK from the three genotypes.

--Fig S4--

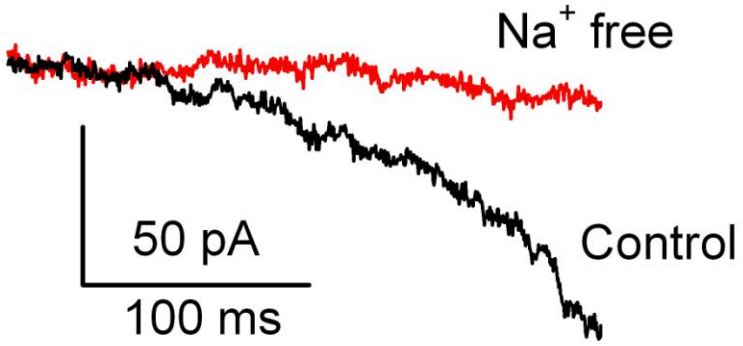

Fig S4 The inward current elicited by ramp protocol was blocked when Na^+ in the bath solution was replaced with Li^+, confirming that the inward currents largely were I_{NCX}.

References

1. Zhang R, Khoo MS, Wu Y, Yang Y, Grueter CE, Ni G, Price EE, Jr., Thiel W, Guatimosim S, Song LS, Madu EC, Shah AN, Vishnivetskaya TA, Atkinson JB, Gurevich VV, Salama G, Lederer WJ, Colbran RJ, Anderson ME. Calmodulin kinase II inhibition protects against structural heart disease. Nat Med. 2005;11:409-417.
2. Khoo MS, Li J, Singh MV, Yang Y, Kannankeril P, Wu Y, Grueter CE, Guan X, Oddis CV, Zhang R, Mendes L, Ni G, Madu EC, Yang J, Bass M, Gomez RJ, Wadzinski BE, Olson EN, Colbran RJ, Anderson ME. Death, cardiac dysfunction, and arrhythmias are increased by calmodulin kinase II in calcineurin cardiomyopathy. Circulation. 2006;114:1352-1359.
3. Gao Z, Chen B, Joiner ML, Wu Y, Guan X, Koval OM, Chaudhary AK, Cunha SR, Mohler PJ, Martins JB, Song LS, Anderson ME. I(f) and SR Ca(2+) release both contribute to pacemaker activity in canine sinoatrial node cells. J Mol Cell Cardiol. 2010;49:33-40.
4. Wu Y, Gao Z, Chen B, Koval OM, Singh MV, Guan X, Hund TJ, Kutschke W, Sarma S, Grumbach IM, Wehrens XH, Mohler PJ, Song LS, Anderson ME. Calmodulin kinase II is required for fight or flight sinoatrial node physiology. Proc.Natl.Acad.Sci.U.S.A. 2009;106:5972-5977.
5. Vinogradova TM, Sirenko S, Lyashkov AE, Younes A, Li Y, Zhu W, Yang D, Ruknudin AM, Spurgeon H, Lakatta EG. Constitutive phosphodiesterase activity restricts spontaneous

beating rate of cardiac pacemaker cells by suppressing local Ca2+ releases. Circ Res. 2008;102:761-769.