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Catecholamine-Independent Heart Rate Increases Require CaMKII

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Abstract

Background—Catecholamines increase heart rate by augmenting the cAMP responsive HCN4 'pacemaker current' (I_f) and/or by promoting inward Na^+/Ca^{2+} exchanger current (I_{NCX}), by a 'Ca^{2+} clock' mechanism in sinoatrial nodal cells (SANCs). The importance, identity and function of signals that connect I_f and Ca^{2+} clock mechanisms are uncertain and controversial, but the multifunctional Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII) is required for physiological heart rate responses to β-adrenergic receptor (β-AR) stimulation. The aim of this stuy is to measure the contribution of the Ca^{2+} clock and CaMKII to cardiac pacing independent of β-AR agonist stimulation.

Methods and Results—We used the L-type Ca^{2+} channel agonist BayK 8644 (BayK) to activate the SANC Ca^{2+} clock. BayK and isoproterenol were similarly effective in increasing rates in SANCs and Langendorff-perfused hearts from WT control mice. In contrast, SANCs and isolated hearts from mice with CaMKII inhibition by transgenic expression of an inhibitory peptide (AC3-I) were resistant to rate increases by BayK. BayK only activated CaMKII in control SANCs, but increased I_{Ca} equally in all SANCs, indicating that increasing I_{Ca} was insufficient and suggesting CaMKII activation was required for heart rate increases by BayK. BayK did not increase I_f or protein kinase A (PKA)-dependent phosphorylation of phospholamban (at Ser16), indicating that increased SANC Ca^{2+} by BayK did not augment cAMP/PKA signaling at these targets. Late diastolic intracellular Ca^{2+} release and I_{NCX} were significantly reduced in AC3-I SANCs and the response to BayK was eliminated by ryanodine in all groups.

Conclusions—The Ca^{2+} clock is capable of supporting physiological fight or flight responses, independent of β-AR stimulation or I_f increases. Complete Ca^{2+} clock and β-AR stimulation responses require CaMKII.

Keywords

Ca^{2+}/calmodulin-dependent protein kinase (CaMKII); sinoatrial node cells; L-type Ca^{2+} channels; pacemaker current; sarcoplasmic reticulum

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Introduction

The fight or flight response to increase heart rate is a fundamental aspect of cardiovascular physiology, but recent findings have unsettled a core concept that increases in heart rate are exclusively due to a cyclic adenosine monophosphate (cAMP)-activated pacemaker current (I_f). Accumulating data support a view that sinoatrial node (SAN) pacemaker automaticity is an outcome of cell membrane depolarization triggered by an inward I_f, conducted primarily by HCN4, and inward Na^+/Ca^{2+} exchange current (I_{NCX}), activated by spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR).[1-4] Increases in I_f and I_{NCX} steepen the slope of phase [4] diastolic depolarization (DD) leading to faster SAN cell (SANC) action potential triggering.[4-5] Catecholamines activate the cAMP and protein kinase A (PKA) signaling pathway by agonist actions at β adrenergic receptors (β-AR), and so have the capability of increasing I_f, SR Ca^{2+} release and I_{NCX}.[6] However, recent work suggests that SANCs also utilize a β-AR independent mechanism for promoting cAMP activity by Ca^{2+}-dependent activation of adenylyl cyclase.[7] Despite the potential interconnections between β-AR-initiated and Ca^{2+} responsive signaling pathways for augmenting I_f and I_{NCX} to increase heart rate, we are unaware of any studies examining the signaling mechanisms engaged by increasing intracellular Ca^{2+}, independent of β-AR agonist stimulation, in SANCs. Isoproterenol, a β-AR agonist, increases intracellular Ca^{2+} in SANCs and activates the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).[8] We recently reported that CaMKII was required for the physiological heart rate response to isoproterenol,[9] but it is unknown if CaMKII actions require β-AR activation or if CaMKII activation in the absence of β-AR activation is sufficient to increase heart rate. We designed our study to distinguish the contributions of Ca^{2+} and β-AR signaling to SAN automaticity and to determine if increases in intracellular Ca^{2+} and CaMKII were capable of increasing SAN rates to a similar extent as β-AR stimulation.

Here we increased SANC intracellular Ca^{2+} directly, independently of β-AR stimulation, by increasing L-type Ca^{2+} current (I_{Ca}) with BayK 8644 (BayK). BayK increased mouse and dog SANC automaticity over a dynamic range that was similar to increases evoked by isoproterenol, suggesting that a Ca^{2+} dependent mechanism is robust and capable of supporting the majority of heart rate increases required for the physiological fight or flight response in small and large animal models.[10-11] BayK increased heart rate and SANC automaticity significantly less in Langendorff-perfused isolated mouse hearts and in isolated SANCs with CaMKII inhibition due to transgenic myocardial expression of a CaMKII inhibitory peptide (AC3-I) compared to WT littermates or transgenic control mice with myocardial expression of an inactive scrambled form of AC3-I (AC3-C). These findings suggested that heart rate responses to increased Ca^{2+} relied on activation of CaMKII. BayK increased I_{Ca} equally in AC3-I, AC3-C and WT SANCs, showing that reduced BayK response in AC3-I SANCs was due to actions 'downstream' to I_{Ca}. The increases in rate by BayK were independent of I_f because I_f was not increased by BayK and was equivalent in AC3-I and control SANCs, suggesting that increasing SANC Ca^{2+} with BayK did not activate a cAMP pathway with access to HCN4. Furthermore, BayK did not increase phosphorylation of a *bona fide* PKA phosphorylation target site on phospholamban (Ser16) in SANCs, suggesting that increasing SANC Ca^{2+} does not lead to a generalized increase in PKA activity. BayK increases in SANC automaticity required SR Ca^{2+} release, because they were eliminated by ryanodine and because AC3-I SANCs failed to show increased frequency of late diastolic SR Ca^{2+} release events or enhanced I_{NCX} in response to BayK, compared to controls. These data show that CaMKII activation, independent of β-AR stimulation, can support heart rate increases over a dynamic range that is similar to the classical β-AR agonist stimulated fight or flight response. CaMKII is required for increasing heart rate by β-AR and Ca^{2+}-based mechanisms in SANCs.

Methods

Methodological details are included in the supplemental materials (available online at http://circep.ahajournals.org/). Single SANCs from mouse and dog were isolated.[9, 12] We recorded APs, I_f and I_{NCX} with perforated patch access, I_{Ca} was recorded with conventional whole celll mode. All voltage and current clamp studies were performed at 36°C. Intracellular Ca^{2+} transients and sparks were measured with a confocal microscope (LSM510, Carl Zeiss MicroImaging) using the line scan mode, as described.[12-13] Western analyses were performed to measure the phosphorylated (Ser16 or Thr17) and total phospholamban expression in SAN tissue. The data are presented as means ± the standard error of the mean (S.E.M,). The statistical significance was evaluated with one-way, two-way ANOVA or Student's *t*-test as appropriate, Student-Newman-Keuls was used for post hoc analysis. $P<0.05$ is considered statistically significant.

Results

Equivalent SAN rate acceleration by BayK and β-AR stimulation

We used a selective L-type Ca^{2+} channel agonist, BayK,[14] to examine if increasing I_{Ca} affected the spontaneous activity of SANCs. The SANCs were perfused for at least 5 mins with different concentrations of BayK. BayK significantly and dose-dependently increased the pacemaking activity of SANCs isolated from WT mice (Fig 1A and B). Interestingly, the rate-dose response relationship to BayK was similar to the rate-dose response relationship observed with isoproterenol (Fig 1B). The maximum effects of BayK and isoproterenol were equivalent (Fig 1C). These findings showed that BayK and isoproterenol were similarly effective in increasing SANC automaticity. We next asked if the rate response to BayK occurred in larger mammals, by repeating our studies with canine SANCs. We found that BayK increased the spontaneous activity in single SANCs isolated from dogs, similar to our findings in mouse SANCs (Supplementary data Fig S1). The rate increase by BayK in dog SANCs was close to what we found with isoproterenol.[12] These data indicate that BayK induces significant rate increases in SANCs isolated from mice and dogs, suggesting that studies of heart rate responses to BayK in mice will also provide information relevant to larger mammals.

CaMKII is required for BayK-triggered increases in SANC rates

We recently reported that CaMKII is required for SAN "fight or flight" physiology during β-AR stimulation.[9] In order to test whether acceleration of SAN automaticity by L-type Ca^{2+} channel activation, independent of β-AR stimulation, requires CaMKII, we measured the effects of BayK on isolated SANCs and Langendorff-perfused hearts from mice with cardiomyocyte-delimited expression of AC3-I. We used AC3-C expressing mice and WT littermates as controls.[15] At baseline, the spontaneous activity was similar among AC3-I, AC3-C and WT SANCs, similar to our earlier report (Fig 2A and B).[9] However, the rate increase in AC3-I SANCs after BayK was less than half of the BayK-triggered rate increase in controls (Fig 2C). These results showed that BayK-triggered increases in automaticity were significantly ($P=0.003$) reduced in AC3-I compared to control SANCs. The rate increases by BayK were not eliminated by AC3-I expression, likely because I_{Ca} contributes directly to DD and because AC3-I inhibition of CaMKII activity is incomplete.[15] Similar to the isolated SANCs, there was no difference in the baseline beating rates in AC3-I, AC3-C and WT Langedorff-perfused hearts. BayK increased the heart rates in all mice, but the increase in AC3-I hearts was significantly ($P=0.005$) less than that in hearts from WT and AC3-C mice (Supplementary data Fig S2). These results indicated that CaMKII is required for the full range of SANC rate response to BayK.

Reduced DDR response but preserved I_f and I_{Ca} in AC3-I SANCs

Modulating the spontaneous DD is the cell membrane potential mechanism for governing SANC rate.[16] At baseline the DD rate (DDR) did not differ among the three genotypes. However, DDR increases by BayK in AC3-I mice were significantly (P=0.01) less than in WT and AC3-C controls (Fig 2D). BayK did not significantly alter MDP (Fig 2E) or APD (data not shown) in any of the groups. Several Ca^{2+} sensitive adenylyl cylases are expressed in SAN, suggesting the possibility that BayK may enhance the activity of the cAMP/PKA pathway.[17-18] We next considered whether I_f was involved in rate increases by BayK, and measured I_f from SANCs before and after BayK. BayK (1 μM) did not significantly affect I_f in any of the groups (Fig 3). These results indicated that I_f was unlikely to contribute to the reduced efficacy of BayK in AC3-I SANCs, and suggested that increasing SANC Ca^{2+} by BayK did not activate cAMP[19] or PKA[20] with access to HCN4. We next measured the PKA phosphorylation site (Ser16) on phospholamban,[21-22] to test for evidence of PKA activation by BayK, at a validated PKA target, in SANCs. We did not detect a significant increase in phospholamban Ser16 phosphorylation with BayK treatment (Supplementary data Fig S3) compared to baseline. We interpret our findings to indicate that BayK increases heart rate by a mechanism that is not significantly determined by the cAMP/PKA signaling pathway in SANCs. We next measured I_{Ca} to determine if the reduced rate response to BayK in AC3-I SANCs was due to reduced I_{Ca} responses in AC3-I compared to control SANCs. Consistent with our previous study,[9] there was no difference in the basal I_{Ca} among the three genotypes (Fig 4). BayK equivalently increased peak and integrated I_{Ca} in each of the genotypes, showing that reduced heart rate responses to BayK in AC3-I mice were independent of BayK actions on I_{Ca}. Our data suggested that the reduced BayK response in AC3-I SANCs was determined by a cellular mechanism that was downstream to I_{Ca}.

Ryanodine prevented the SANC response to BayK

Ca^{2+} influx via I_{Ca} is crucial for filling sarcoplasmic reticulum (SR) Ca^{2+} stores and sustaining the capacity for the local Ca^{2+} release events from SR that are thought to contribute to SANC DDR and automaticity.[4, 9] Because CaMKII promotes SANC SR Ca^{2+} release in response to isoproterenol,[9] we hypothesized that local SR Ca^{2+} release events may underlie the rate increases by BayK. We used ryanodine, a SR Ca^{2+} release antagonist, to test if BayK increases would persist in the absence of increases in local SR Ca^{2+} release events. Ryanodine (2 μM) reduced spontaneous automaticity equally in SANCs from AC3-I, AC3-C and WT mice(Fig 5). The SANC rate increases by BayK were significantly and equivalently blunted in all genotypes, suggesting that SR Ca^{2+} release was essential for the differential effects of BayK on pacemaking activity in AC3-I compared to AC3-C and WT SANC.

CaMKII inhibition suppressed SR Ca^{2+} release and BayK triggered increases in I_{NCX}

As SR Ca^{2+} release appeared to contribute to the effects of BayK on SANCpacemaking activity, we acquired confocal Ca^{2+} images to directly measure SR Ca^{2+} release events. BayK increased Ca^{2+} transient amplitudes to a similar level in SANCs from each of the three genotypes (Fig 6A-B). In contrast, BayK significantly increased the frequency of late diastolic local Ca^{2+} release events in SANCs from WT and AC3-C but not AC3-I mice (Fig 6C). However, Ca^{2+} spark amplitude, duration and width remained unaltered by BayK in any of the genotypes (data not shown). These results suggested that the reduced response of AC3-I SANCs stimulated with BayK was due to impaired diastolic SR Ca^{2+} release, similar to the reduction in isoproterenol-stimulated SR Ca^{2+} release in AC3-I SANCs.[9]

Diastolic SR Ca^{2+} release enhances I_{NCX} in SANCs, and the increase in *I*NCX is a critical for promoting DDR.[4, 23-24] We next measured inward currents elicited with a voltage clamp ramp protocol from -60 to -45 mV, mimicking voltage range of spontaneous DD in

SANCs, [25] at baseline and after BayK. The inward currents evoked by the voltage command protocol were predominantly I_{NCX}, because they were nearly eliminated by replacing Na^+ with Li^+ in the bath solution (Supplementary data Fig S4). We found BayK increased inward I_{NCX} in WT and AC3-C significantly more than in AC3-I mice (Fig 7). These results supported the concept that CaMKII promoted increased diastolic Ca^{2+} release to enhance I_{NCX} and heart rate in response to BayK.[25-26]

BayK enhanced CaMKII activity in SANCs

CaMKII becomes constitutively active in response to prolonged Ca^{2+} exposure[27] by autophosphorylation of a threonine residue (Thr 286 or 287 depending on the isoform) in the CaMKII regulatory domain.[28] Our data up to this point strongly supported a concept where CaMKII activation increased DDR and SANC rates by enhancing SR Ca^{2+} release and I_{NCX}. In order to assess the affects of BayK on SANC CaMKII activation, we measured threonine autophosphorylation (p-CaMKII) at baseline and during BayK (Fig 8). p-CaMKII was readily detected after BayK in WT and AC3-C, but not in AC3-I SANCs. We found that p-CaMKII was enriched in a subsarcolemmal distribution after BayK in control SANCs. In contrast, pCaMKII was not evident in SANCs from any of the mice without BayK, under our experimental conditions. We found that BayK significantly increased phosphorylation of a *bona fide* CaMKII site on phospholamban (pThr17) in WT and AC3-C but not in AC3-I mice (supplementary data Fig S3). Taken together, these results show that BayK activates CaMKII under conditions permissive for increased SR Ca^{2+} release and SANC rate increases and that AC3-I expression in SANCs is sufficient to inhibit BayK triggered increases in CaMKII activity.

Discussion

The discovery of cAMP-gated channels[29] was a major milestone toward developing a molecular understanding of mechanisms for physiological cardiac pacing. The critical importance of I_f to cardiac pacing in humans was highlighted by findings that patients with defects in I_f due to mutations in the gene encoding HCN4 required surgical implantation of artificial pacemakers.[30] Recent data shows that heart failure patients benefit from new and highly selective I_f antagonist drugs by reducing heart rate,[31] suggesting that hyperactivation of I_f may contribute to heart disease in humans. We interpret the wealth of cellular and animal studies, in combination with the aforementioned clinical investigations, to provide incontrovertible evidence that I_f is a fundamental mechanism for cardiac pacing. HCN4 is the predominant cAMP gated channel for I_f in SANC [32] and new studies show that HCN4 is also activated by PKA phosphorylation.[20] Thus, β-AR agonist stimulation increases heart rate in part by enhancing SAN cAMP and PKA activity and increasing I_f. However, mice with HCN4 knock out show a surprisingly mild phenotype and have normal or near normal heart rate increases in response to isoproterenol.[33] These and other findings[9, 12, 34] support a view that HCN4-alternative mechanisms also support physiological cardiac pacing.

In this study we activated SANC automaticity by engaging the 'Ca^{2+} clock' mechanism,[4] independently of β-AR activation or I_f by using BayK. We found that BayK and isoproterenol increased SANC rates over a similar dynamic range, suggesting that the Ca^{2+}-based mechanisms for cardiac pacing are capable of supporting a major component of fight or flight heart rate increases. Although the Ca^{2+} clock is recruited in SANC by β-AR agonist stimulation under physiological conditions, our study showed that the Ca^{2+} clock can be activated even in the absence of β-AR receptor stimulation. Increased intracellular Ca^{2+} may increase PKA activity in SANCs due to the relatively enhanced activity of Ca^{2+}-activated adenylyl cyclase isoforms, principally types 1 and 8.[7, 18] The potential for Ca^{2+}-activated signaling molecules to affect I_f has been posited as a mechanism for Ca^{2+} evoked increases in SAN automaticity.[3, 35] Thus, it is possible that BayK-induced increases in SANC

automaticity could be a consequence of increased I_f. However, we found no evidence that BayK evoked increases in cellular Ca^{2+} entry affected I_f. We believe our results provide strong evidence that the Ca^{2+} clock is an independent cellular mechanism that can be clearly distinguished from I_f.

SANCs show increased basal PKA activity compared to atrial and ventricular myocytes,[36] and increased PKA activity is hypothesized to play a critical role in promoting the SR Ca^{2+} release and inward I_{NCX}. [26, 37] Thus, it is possible that hyperactivation of the cAMP/PKA pathway in SANCs, probably due to a relative abundance of Ca^{2+} activated adenylyl cyclase isoforms, could enhance coupling of the I_f and Ca^{2+} clock cellular pacing mechanisms. Several studies now provide evidence showing that β-AR agonist stimulation increases heart rate by coordinate activation of I_f and a Ca^{2+} clock mechanism.[4-5] Our study contributes new information by showing that the Ca^{2+} clock can produce significant increases in heart rate and SANC automaticity by a mechanism requiring CaMKII in the absence of β-AR agonist stimulation. We did not identify evidence for Ca^{2+}-activated cAMP or PKA to increase I_f or to phosphorylate phospholamban. Thus, we interpret our results to show that the Ca^{2+} clock is highly dependent on CaMKII, while Ca^{2+} activated increases in cAMP and PKA signaling to I_f and phospholamban are dispensable, at least under these experimental conditions. The existence of a Ca^{2+} based mechanism for SANC pacing and the relative importance of I_f and SR Ca^{2+} release have become highly polemic issues, with some investigators denying the potential for a Ca^{2+}-dependent, I_f independent mechanism to drive SANC automaticity.[3] We believe that our findings provide new clarity to this controversy, by showing the Ca^{2+} clock, in the absence of β-AR agonist stimulation, to be a highly effective cellular mechanism for SANC and cardiac pacing. In this study we used BayK as an experimental tool to interrogate the Ca^{2+} clock. Under physiological conditions, our results suggest that Ca^{2+} clock activation by enhanced CaMKII with β-AR stimulation may at least partially accout for the heart rate increase in the fight or flight response.

CaMKII now appears to be an essential component of SANC automaticity due to β-AR agonists and BayK. As such, CaMKII represents a highly adaptable connection between I_f and SR-based SANC pacing mechanisms. CaMKII increases $Ca_V1.2$ current and phosphorylation of $Ca_V1.2$ channel β subunits, which are presumably shared by $Ca_V1.3$, leads to mode 2 gating,[38] a high activity state originally identified as a response to BayK.[39] By using BayK we were able to circumvent the potentially confounding effects of CaMKII on SANC Ca^{2+} entry by I_{Ca}. Surprisingly, BayK activation of I_{Ca}, in the presence of reduced CaMKII activity or impaired SR Ca^{2+} release, was insufficient to significantly increase SANC automaticity. In contrast, CaMKII activity and SR Ca^{2+} release coupled to BayK-enhanced I_{Ca} effectively increased SANC rates by diastolic SR Ca^{2+} release favoring DD due to inward I_{NCX}. Although BayK failed to increase diastolic SR Ca^{2+} release and consequent I_{NCX} in AC3-I mice, BayK resulted in modest enhancement of SANC automaticity, suggesting that the increases of I_{Ca} by BayK also directly contribute to phase 4 DD. SR Ca^{2+} release is essential for myofilament activation during excitation-contraction coupling in myocardium, whereas increased diastolic SR Ca^{2+} release is associated with arrhythmias and myopathy in ventricular and atrial myocardium. CaMKII hyperactivation, perhaps partly as a response to increased SR Ca^{2+}, is now recognized to contribute to cardiomyopathy and arrhythmias.[40-41] In contrast, diastolic SR Ca^{2+} leak and CaMKII activation appear to be fundamental aspects of the physiological function of SANC. It will be interesting to learn if hyperactivation of CaMKII in SANCs leads to defects in cardiac pacing, and if CaMKII inhibition could be used therapeutically to prevent excessive SANC firing rates in heart failure, similar to the recent success of I_f antagonists.

A once sleepy backwater in cardiac biology, the mechanisms of cardiac pacing have become interesting and controversial. Two competing paradigms for cardiac pacing have

vied for hegemony: an HCN4 directed pacemaker current (I_f) and a Ca^{2+} clock mechanism where the cellular Ca^{2+} handling apparatus supporting excitation-contraction coupling in atrium and ventricle has been repurposed for sinoatrial nodal cell automaticity. Some research appears to support a view that the Ca^{2+} clock and I_f mechanisms are inter-related by Ca^{2+} activation of cAMP/PKA signaling. In every case, the starting point for studying these mechanisms has been β-adrenergic receptor (β-AR) agonist ligands. Here we employed Bay K8644, a selective agonist of L-type Ca^{2+} channel, to activate the Ca^{2+} clock in the absence of catecholamines. We found the Ca^{2+} clock, in the absence of β-AR agonist stimulation or evidence of cAMP/PKA activation, is a highly effective cellular mechanism for SANC and cardiac pacing and that the Ca^{2+} clock and β-AR stimulation responses require CaMKII. It will be interesting to learn if hyperactivation of CaMKII in SANCs leads to defects in cardiac pacing, and if CaMKII inhibition could be used therapeutically to prevent excessive SANC firing rates in heart failure, similar to the recent success of I_f antagonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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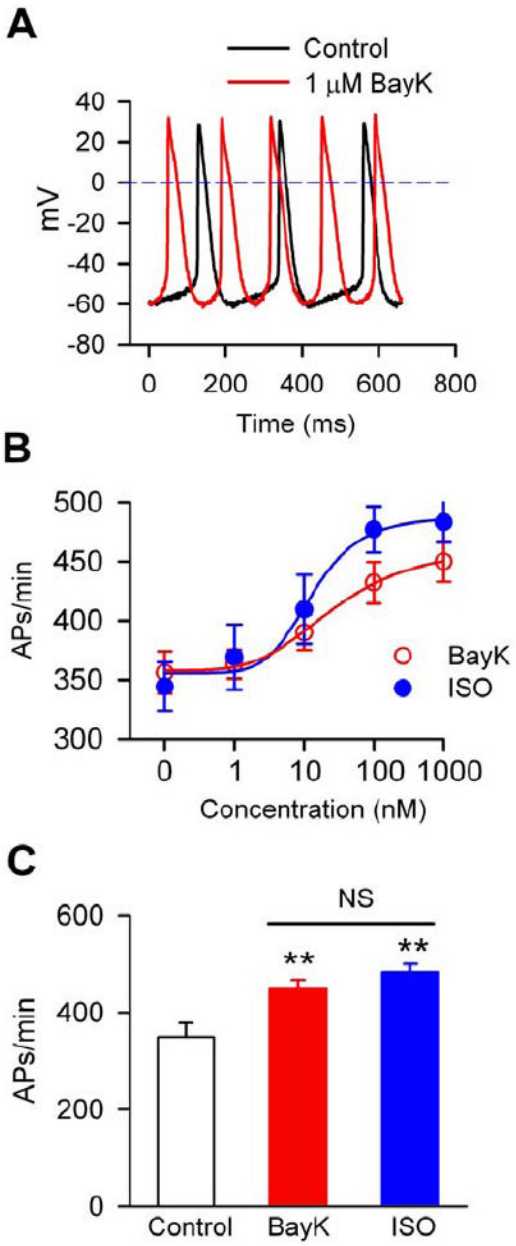

Fig 1.

BayK increased the pacemaking activity of SANCs. **A.** Representative AP traces in a mouse SANC before (black line) and after (red line) 1 μM BayK. **B.** Dose-response curves of BayK and isoproterenol on the automaticity of mouse SANCs, n=5-22 for each dose. **C.** Summary data of SANC spontaneous activity under control conditions (n=28), in the presence of 1 μM BayK (n=24) and 1 μM isoproterenol (ISO, n=5) One-way ANOVA **P<0.01 vs control.

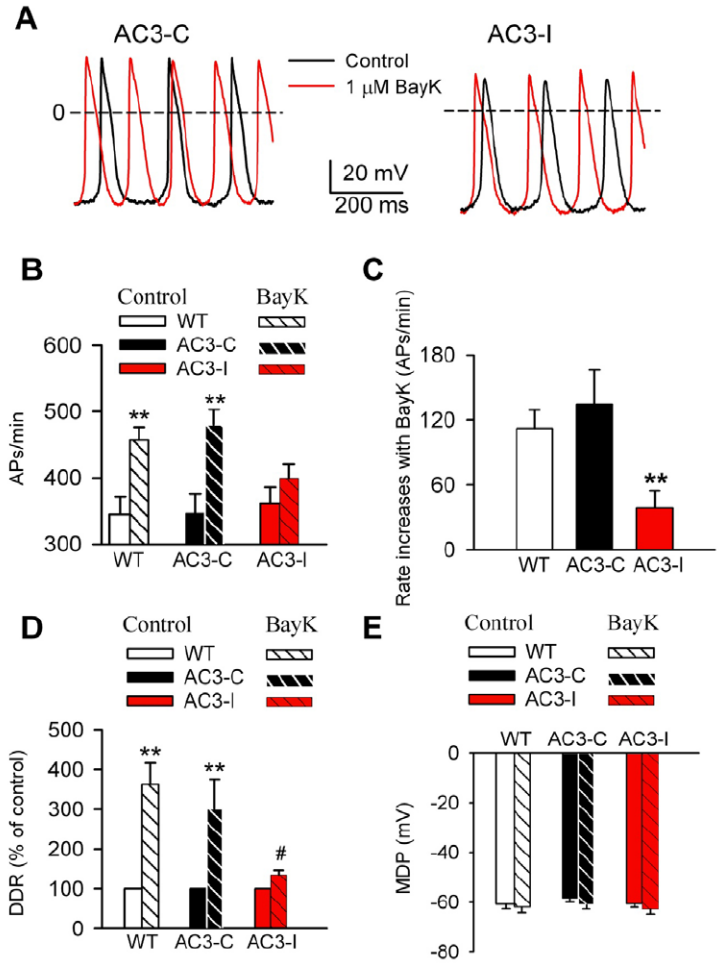

Fig 2.

SANC rate increases by BayK are reduced by CaMKII inhibition. **A.** Representative AP traces recorded before and after BayK in SANCs isolated from AC3-C and AC3-I mice. **B.** Spontaneous activity of SANCs in the absence and presence of 1 μM BayK. **P<0.01 vs control, n≥16 cells from more than 4 animals for each group. **C.** SANC rate increases by BayK in WT, AC3-C and AC3-I mice (data from panel B). ** P<0.001 vs WT or AC3-C, n≥16 cells from more than 4 animals for each group. **D.** BayK (1 μM) significantly increased the DDR in SANCs from WT and AC3-C, but not AC3-I mice. **P<0.01 vs control. # P<0.05 vs WT and AC3-C mice, n≥16 cells from more than 4 animals for each group. **E.** BayK did not significantly alter the MDP in any groups (data from panel D), n≥16 cells from more than 4 animals for each group.

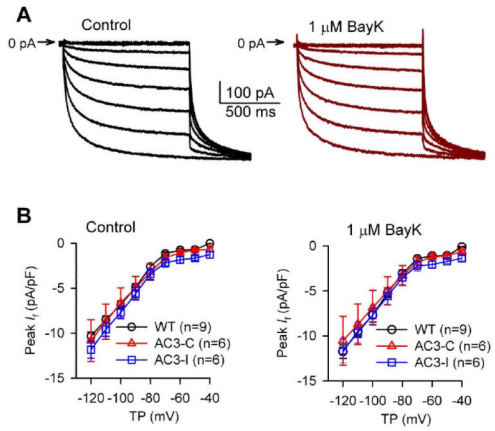

Fig 3.

BayK did not affect I_f. **A.** Representative I_f traces recorded in a SANC isolated from a WT mouse before and after 1 μM BayK. **B.** I-V relationship of I_f under control conditions and in the presence of 1 μM BayK.

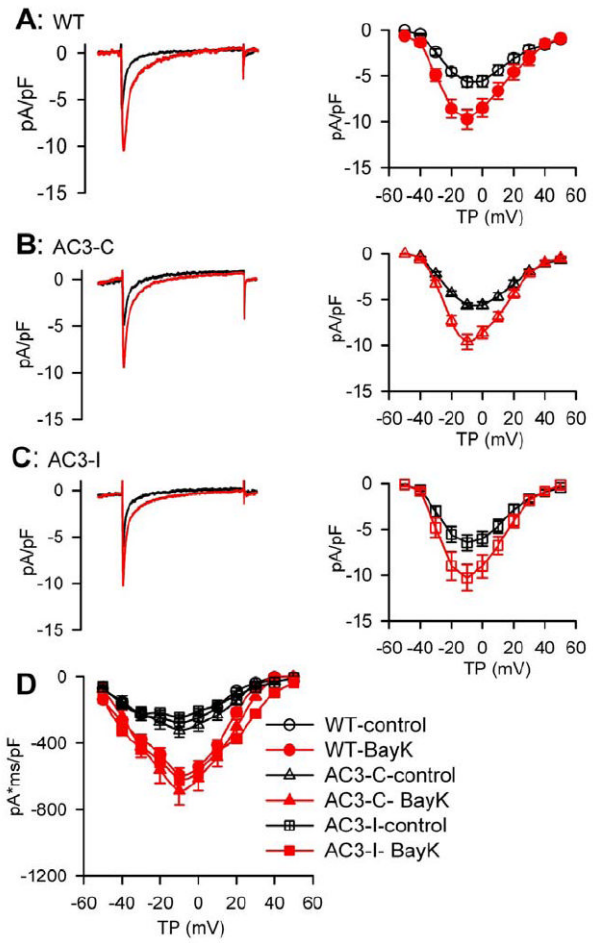

Fig 4.

BayK equally increased I_{Ca} in AC3-I, AC3-C and WT SANCs. **A-C.** representative I_{Ca} traces (left panels) and I-V relationship (right panels) of peak currents before and after 1 μM BayK from WT (**A**), AC3-C (**B**) and AC3-I (**C**) mice. **D.** The integrated I_{Ca} I-V relationship from WT, AC3-C and AC3-I SANCs, under control conditions and in the presence of BayK, n=8-19 cells from more than 3 animimals for each group.

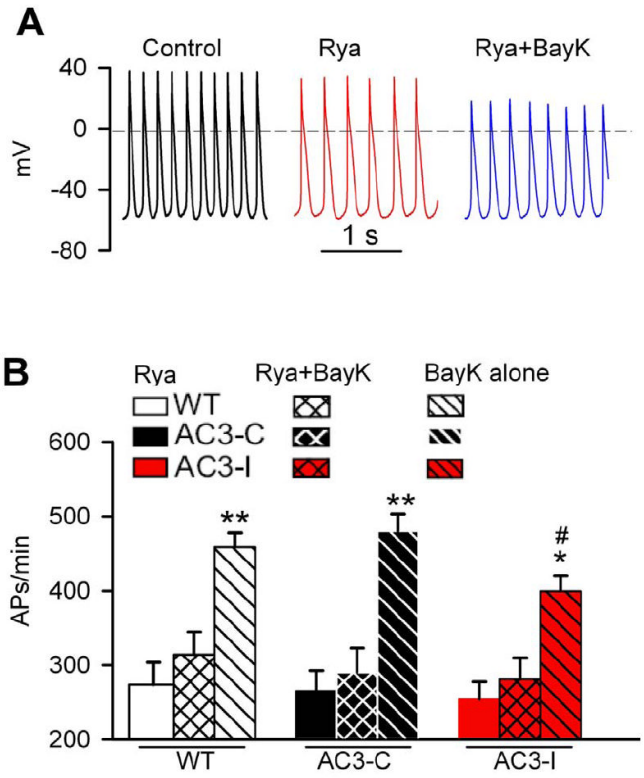

Fig 5.

Ryanodine (Rya) pretreatment prevented BayK triggered SANC rate increases. **A.** Representative APs in control, in the presence of Rya and the combination of Rya plus BayK. **B.** SANC spontaneous pacemaking activity in the presence of Rya, the combination of Rya plus BayK, and BayK alone. *P<0.05 vs Rya and Rya+BayK, **P<0.001 vs Rya and Rya+BayK, #P<0.05 vs WT and AC3-C in the presence of BayK. n=7-10 cells from 3 animals for each genotypes.

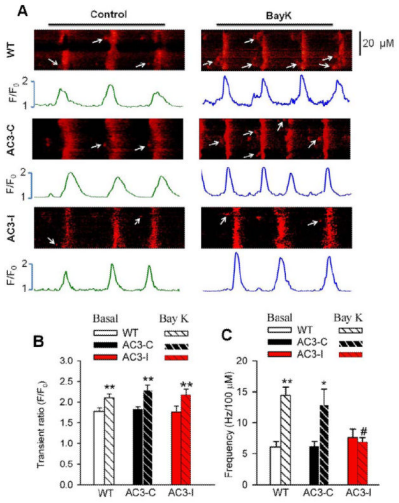

Fig 6.

Effect of BayK on the SANC intracellular Ca^{2+}. **A.** Representative Ca^{2+} images in control and in the presence of 1 μM BayK. The arrows indicate late diastolic Ca^{2+} sparks. **B.** Summary data for Ca^{2+} transients. n=10-35 cells from more than 5 animals for each group. **P<0.01 vs control. **C.** Summary data for Ca^{2+} spark frequency. *P<0.05, vs control, **P<0.01 vs control, #P<0.05 vs WT and AC3-C mice. n≥18 cells from more than 4 animals for each group.

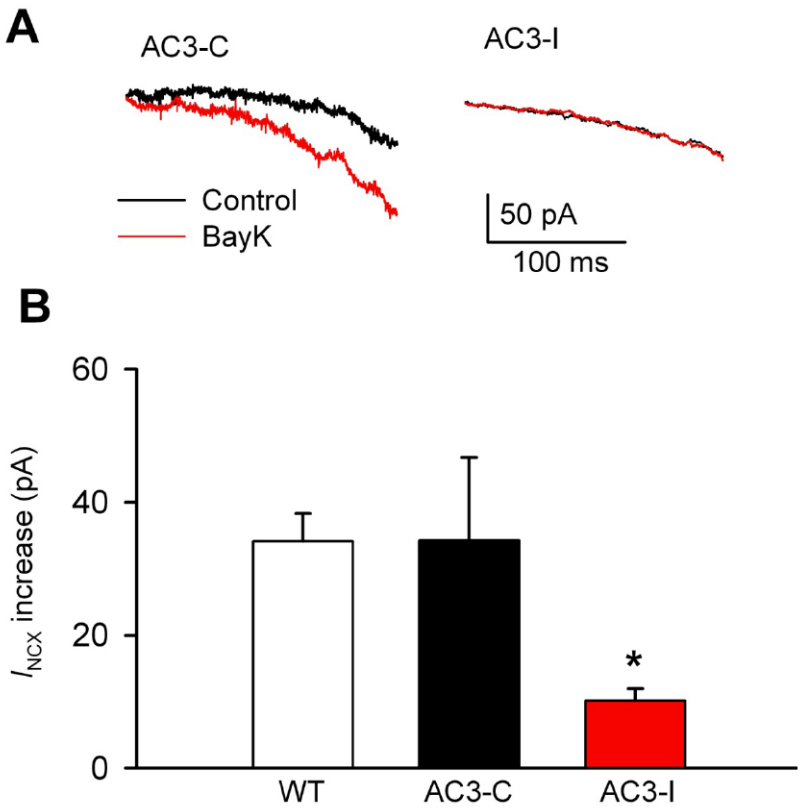

Fig 7.

Reduced I_{NCX} response to BayK in AC3-I SANCs. **A.** Representative SANC I_{NCX} traces recorded from AC3-C and AC3-I mice before and after 1 μM BayK. **B.** I_{NCX} increases by BayK. **P<0.05 vs WT or AC3-C mice, n=5-10 cells from 3 animals for each group.

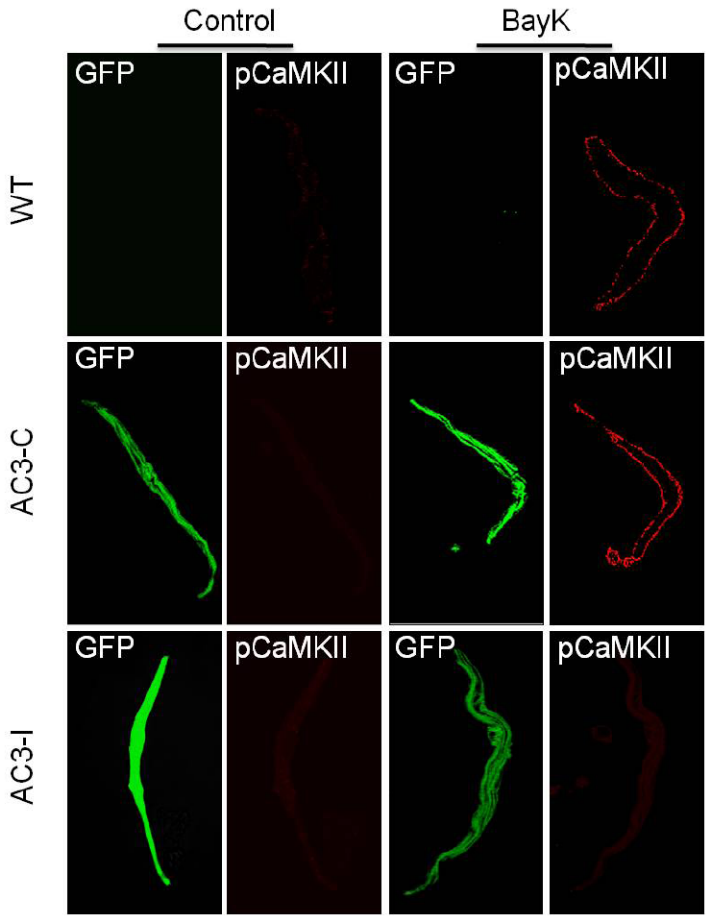

Fig 8.

AC3-I expression eliminated CaMKII autophosphorylation response to BayK in mouse SANCs. Representative immunofluorescence images show BayK enhanced CaMKII autophosphorylation (pCaMKII) in SANCs isolated from WT and AC3-C, but not AC3-I mice. Note: eGFP is expressed in AC3-C and AC3-I SANCs. *P<0.05 vs WT and AC3-C