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Ca²⁺-Dependent Phosphorylation of Ca²⁺ Cycling Proteins Generates Robust Rhythmic Local Ca²⁺ Releases in Cardiac Pacemaker Cells

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Abstract

The spontaneous beating of the heart is governed by spontaneous firing of sinoatrial node cells, which, unlike primary contractile ventricular myocardial cells, generate action potentials due to spontaneous depolarization of the membrane potential, or diastolic depolarization. The spontaneous diastolic depolarization rate is determined by spontaneous local submembrane Ca²⁺ releases through ryanodine receptors (RyRs). We sought to identify specific mechanisms of intrinsic Ca²⁺ cycling by which sinoatrial node cells, but not ventricular myocytes, generate robust, rhythmic local Ca²⁺ releases. At similar physiological intracellular Ca²⁺ concentrations local Ca²⁺ releases were large and rhythmic in permeabilized sinoatrial node cells, but were small and random in permeabilized ventricular myocytes. Furthermore, sinoatrial node cells spontaneously released more Ca²⁺ from the sarcoplasmic reticulum than did ventricular myocytes, despite comparable sarcoplasmic reticulum Ca²⁺ content in both cell types. This ability of sinoatrial node cells to generate more robust and rhythmic local Ca²⁺ releases was associated with increased abundance of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), reduced abundance of the SERCA inhibitor phospholamban, and increased Ca²⁺-dependent phosphorylation of phospholamban and RyR. The increased phosphorylation of RyR in sinoatrial node cells may facilitate Ca²⁺ release from the sarcoplasmic reticulum, whereas Ca²⁺-dependent increase in phosphorylation of phospholamban relieves its inhibition of SERCA, augmenting the pumping rate of Ca²⁺ required to support robust, rhythmic local Ca²⁺ releases. The differences in Ca²⁺ cycling between sinoatrial node cells and ventricular myocytes provide insights into the regulation of “clock-like” intracellular Ca²⁺-cycling that drives normal automaticity of sinoatrial node cells.

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INTRODUCTION

The heart constantly supplies blood to the body by contracting ninety thousand beats per day, and more than 2 billion times during a human lifespan. The heart beat is initiated in the sinoatrial node, the primary physiological pacemaker of the heart in which cells constantly and spontaneously generate action potentials that govern the rate of contraction of the entire heart. In humans, Ca^{2+} release through ryanodine receptors (RyRs), which are sarcoplasmic reticulum (SR) Ca^{2+} release channels, has a key role in cardiac pacemaking. Mutations of RyRs are associated with a deterioration of human sinoatrial node function, atrial fibrillation and atrial standstill (1). The elucidation of the features of Ca^{2+} cycling in cardiac pacemaker cells not only enhances our understanding of how cells of the sinoatrial node function, but also provides new insights into therapies for various types of sinus node dysfunctions (2).

Spontaneous activity of sinoatrial node cells, which distinguishes them from ventricular myocytes, originates from spontaneous diastolic depolarization of the cell surface membrane which gradually grows in amplitude during diastole, driving the membrane potential to the threshold to fire a spontaneous action potential (3). Sarcolemmal ion channel currents are critical in generating diastolic depolarization, including a hyperpolarization activated current I_f , a delayed rectifier potassium current I_K , and L-type and T-type Ca^{2+} currents (3).

Spontaneous subsarcolemmal local Ca^{2+} releases through RyRs are also critical for the generation of diastolic depolarization (4). Local Ca^{2+} releases in sinoatrial node cells occur during diastolic depolarization, preceding the next action potential upstroke, and activate an inward Na^+ - Ca^{2+} exchange (NCX) current which exponentially increases terminal diastolic depolarization (4, 5). The local Ca^{2+} release period, or the time elapsing from the prior action potential triggered SR Ca^{2+} transient to local Ca^{2+} release occurrence, regulates the spontaneous beating rate of sinoatrial node cell by delineating the timing of NCX activation and thus prompting the next action potential (6). Rhythmic local Ca^{2+} releases in sinoatrial node cells reflect rhythmic Ca^{2+} cycling by the SR, which has been dubbed the “ Ca^{2+} clock”. Specifically, action potential-induced Ca^{2+} depletion of the SR and RyR inactivation are followed by refilling of the SR Ca^{2+} load and recovery of RyR activation. The local Ca^{2+} release period critically depends on both the recovery of RyRs from inactivation and the rate of Ca^{2+} replenishment of the SR, which is controlled by the SR Ca^{2+} ATPase (SERCA) (7). Spontaneous Ca^{2+} release events originating from RyRs called “ Ca^{2+} sparks” have initially been discovered in ventricular myocytes as discrete regions of increased fluorescence produced by bursts of Ca^{2+} (8). In contrast to spontaneous local Ca^{2+} releases in sinoatrial node cells, Ca^{2+} sparks in ventricular myocytes are relatively small, occur stochastically and produce small local depletions of SR Ca^{2+} , which is thought to be rapidly replenished through diffusion from neighboring, extensively interconnected regions in the SR, rather than by reuptake of Ca^{2+} by the SR (9, 10). Specific mechanisms that allow sinoatrial node cells, but not ventricular myocytes, to generate large and rhythmic local Ca^{2+} releases through RyRs in the basal state remain unclear. The cardiac SR oscillates Ca^{2+} through the same proteins in rabbit sinoatrial node cells and ventricular myocytes, SERCA and RyRs (11). The kinetics of Ca^{2+} pumping in the SR is primarily defined by the activity of SERCA. Mice with a cardiomyocyte-specific disruption of the *SERCA* gene and ~50% reduced abundance of SERCA show a 2-fold reduction in Ca^{2+} wave generation in ventricular

myocytes compared with control mice (12). The activity of SERCA is inhibited by phospholamban (PLB), which in the dephosphorylated state binds to SERCA (13). When PLB is phosphorylated by protein kinase A (PKA) or Ca^{2+} /calmodulin-dependent kinase II (CaMKII), it dissociates from SERCA relieving its inhibition and thus enhancing the Ca^{2+} pumping rate of SERCA into the SR (13). Treating permeabilized ventricular myocytes with an antibody that recognizes PLB and disrupts its interaction with SERCA disrupts PLB-dependent suppression of SERCA activity (14) and increases the number and size of Ca^{2+} sparks (15).

The distribution of RyR, which are Ca^{2+} release channels of the SR, within sinoatrial node cells resembles that in ventricular myocytes, with RyR located both beneath sarcolemma and in the interior of the cell (11, 16, 17). Within sinoatrial node cells RyRs are localized in transverse bands spaced $\sim 2 \mu\text{m}$ apart (17), an interval similar to the sarcomere spacing in ventricular myocytes. The highest density of RyR is found beneath sarcolemma of sinoatrial node cells, which exceeds that in ventricular myocytes by almost 3-fold (11).

Considering the different sets of surface membrane ion channels in sinoatrial node cells and ventricular myocytes and the regulation of many of these channels by Ca^{2+} (3), intracellular Ca^{2+} cycling cannot be explicitly compared in intact sinoatrial node cells and ventricular myocytes. Cell surface membrane permeabilization has been used to study intracellular Ca^{2+} cycling in cardiac cells and has revealed the mechanism of Ca^{2+} -induced release of Ca^{2+} (CICR) from the SR in cardiac cells (18). When the cell surface membrane is permeabilized to remove membrane currents, Ca^{2+} cycling by the SR becomes “free running” and is controlled mostly by the concentration of free cytosolic Ca^{2+} and the kinetics of Ca^{2+} pumping into and release from the SR.

We assessed Ca^{2+} cycling in permeabilized sinoatrial node cells and ventricular myocytes under controlled conditions and similar free cytosolic Ca^{2+} concentrations to determine the differences in subsarcolemmal Ca^{2+} releases in sinoatrial node cells and ventricular myocytes at a steady concentration of cytosolic Ca^{2+} ; how variations of cytosolic Ca^{2+} modulate spontaneous Ca^{2+} releases in the two cell types; and the Ca^{2+} cycling mechanisms that differ in pacemaker cells and ventricular myocytes.

Our results showed that at the same physiological cytosolic Ca^{2+} concentration, sinoatrial node cells cycled Ca^{2+} beneath sarcolemma more efficiently than ventricular myocytes. Specifically, sinoatrial node cells could sustain larger and more rhythmic spontaneous SR Ca^{2+} releases than ventricular myocytes at similar amounts of Ca^{2+} in the SR, which correlated with increased abundance of SERCA and reduced abundance of PLB. Moreover, in response to increased concentrations of cytosolic Ca^{2+} , phosphorylation of PLB and RyR by PKA and CaMKII was increased to a greater extent in sinoatrial node cells than in ventricular myocytes. We speculate that the resulting phosphorylation-dependent modulation of Ca^{2+} pumping in and release from the SR in sinoatrial node cells permitted robust, controlled, spontaneous Ca^{2+} releases by RyRs that were linked to the pacemaker nature of sinoatrial node cells.

RESULTS

Spontaneous local Ca^{2+} releases were more robust and periodic in sinoatrial node cells than in ventricular myocytes

Representative subsarcolemmal linescan images demonstrated that at the same free cytosolic Ca^{2+} concentration, spontaneous local Ca^{2+} releases in permeabilized sinoatrial node cells were comparatively large clustered events, whereas spontaneous Ca^{2+} releases in ventricular myocytes (Ca^{2+} sparks) were significantly smaller (Fig. 1A–D, Fig. S1A). Because the number of Ca^{2+} releases within a given time and space does not adequately reflect the total amount of Ca^{2+} released from the SR by either cell type, we estimated the total Ca^{2+} signal mass released by sinoatrial node cells or ventricular myocytes by integrating signal masses of all spontaneous Ca^{2+} releases within a given time and space of the linescan image (6). Sinoatrial node cells and ventricular myocytes released comparable total Ca^{2+} signal masses at relatively low cytosolic Ca^{2+} concentrations (50–100 nmol/L). When the cytosolic Ca^{2+} concentration was increased above 150 nmol/L, the total Ca^{2+} signal mass released by sinoatrial node cells was ~2–3-fold larger than that released by ventricular myocytes (Fig. 1E). Because diastolic free Ca^{2+} concentrations are between ~160–250 nmol/L in intact spontaneously beating rabbit sinoatrial node cells and paced ventricular myocytes (6, 16, 19), these concentrations of free cytosolic Ca^{2+} in permeabilized cells are physiologically relevant.

Subsarcolemmal local Ca^{2+} releases in sinoatrial node cells were also rhythmic (Fig. 1A), whereas sparks in ventricular myocytes occurred randomly (Fig. 1C). To study the periodicity of spontaneous Ca^{2+} releases in sinoatrial node cells or ventricular myocytes we employed fast Fourier transform (FFT) (20), which transforms signals in the time domains (Fig. 1A and Fig. 1C) into their frequency domain representations (Fig. 1B and Fig. 1D). For comparable analysis Ca^{2+} waveforms were calculated from consecutive 2–3 μm strips of linescan image, which was the average spark size. A single peak in the frequency domain in sinoatrial node cells (Fig. 1B) indicated that spontaneous Ca^{2+} releases appeared repeatedly after approximately the same time-interval and thus were periodic. In contrast, multiple uniformly distributed peaks in the frequency domain in ventricular myocytes (Fig. 1D) indicated a lack of periodicity in spontaneous Ca^{2+} releases. Sinoatrial node cells, but not ventricular myocytes, generated periodic spontaneous Ca^{2+} releases at relatively low cytosolic Ca^{2+} concentrations (50–150 nmol/L) (Fig. 1A, Fig. 1F). Increasing the cytosolic Ca^{2+} concentration resulted in increases in the Ca^{2+} release size (Fig. S1A) and the number of cells with periodic Ca^{2+} releases in both cell types (Fig. 1F, Fig. S2). Ca^{2+} releases in ventricular myocytes became periodic when their size reached ~4 μm (Fig. S2), and the size of spontaneous Ca^{2+} releases correlated with the number of cells generating periodic Ca^{2+} releases (Fig. S2).

Amplified Ca^{2+} release from the SR in sinoatrial node cells was not attributable to increased Ca^{2+} content in the SR

We compared the Ca^{2+} load of the SR in permeabilized sinoatrial node cells and ventricular myocytes by applying a rapid pulse of caffeine, which binds to RyRs and causes them to open, thereby emptying Ca^{2+} stores of the SR (16). The caffeine-releasable Ca^{2+} content in

the SR was the same in both cell types over a broad range of physiologically relevant free cytosolic Ca^{2+} concentrations (50–250 nmol/L) (Fig. 2, A–C). Thus, sinoatrial node cells could sustain larger, rhythmic spontaneous Ca^{2+} releases through RyRs at similar Ca^{2+} loads in the SR as ventricular myocytes. When cytosolic Ca^{2+} was further increased to 300 nmol/L spontaneous local Ca^{2+} releases in sinoatrial node cells disappeared (Fig. S3A) as SR Ca^{2+} load decreased (Fig. 2C). In contrast, increasing the cytosolic Ca^{2+} concentration to 300 nmol/L or greater increased the Ca^{2+} content of the SR in ventricular myocytes (Fig. 2C) leading to the appearance of rhythmic Ca^{2+} wavelets (Fig. S2, Fig. S3).

Increased Ca^{2+} releases through RyRs in sinoatrial node cells in the absence of detectable depletion of Ca^{2+} in the SR suggested that Ca^{2+} pumping into the SR through SERCA was increased. Western blots of SERCA in tissue homogenates demonstrated that abundance of SERCA in rabbit sinoatrial node and atrium exceeded that in ventricle by ~1.5 and ~2-fold, respectively (Fig. 3A). The Western blot analysis was repeated in isolated cells to exclude the possibility of contamination from other cell types and confirmed that the abundance of SERCA in sinoatrial node cells surpassed that in ventricular myocytes by ~1.5-fold (Fig. 3B). In addition, the abundance of PLB in sinoatrial node cells was ~2-fold less than that in ventricular myocytes (Fig. 3C) suggesting that inhibition of SERCA by PLB could be lower in sinoatrial node cells than in ventricular myocytes.

Increased cytosolic Ca^{2+} concentration resulted in increased phosphorylation of PLB in cardiac pacemaker cells

Phosphorylation of PLB disengages it from SERCA, thereby relieving the inhibition of SERCA and increasing its Ca^{2+} pumping rate (13, 16). We assessed the phosphorylation status of PLB at Ser¹⁶, a PKA targeted site. Increased cytosolic Ca^{2+} concentrations might stimulate Ca^{2+} -activated adenylyl cyclases in sinoatrial node cells (21–22), and increase cAMP concentrations and cAMP-mediated PKA-dependent phosphorylation (23). Indeed, an increase in the cytosolic Ca^{2+} concentration resulted in increases in both the cAMP concentration (Fig. 4A) and the phosphorylation of Ser¹⁶ in PLB (Fig. 4B) in permeabilized sinoatrial node cells, but did not alter the phosphorylation of Ser¹⁶ in PLB in ventricular myocytes (Fig. 4C).

Ca^{2+} also activates CaMKII (24–25), which phosphorylates PLB at Thr¹⁷ (13,16) and RyR (26–27). An increase in the cytosolic Ca^{2+} concentration was associated with a significant increase in the phosphorylation of PLB at Thr¹⁷ in sinoatrial node cells (Fig. S4), but not in ventricular myocytes (Fig. S4 and S5). The CaMKII inhibitor KN-93, but not its inactive analog KN-92, suppressed phosphorylation of PLB at Thr¹⁷ in sinoatrial node cells (Fig. S4C).

Ca^{2+} -dependent phosphorylation of RyRs was associated with amplified Ca^{2+} release from RyRs

Phosphorylation of RyR2 is a key mechanism to alter RyR2 activity and Ca^{2+} release from the SR, though the exact effects of phosphorylation on RyR function are unclear (16,26–29). Cardiac RyRs in ventricular myocytes are phosphorylated by both PKA and CaMKII at Ser²⁸⁰⁸ (in mice) or Ser²⁸⁰⁹ (in rabbit) (27,30), which increases the activation of RyRs by

cytosolic Ca^{2+} and enhances RyR Ca^{2+} release (16,26,28). Mice with a form of RyR2 that cannot be phosphorylated by PKA at Ser²⁸⁰⁸ have reduced basal heart beating rates and blunted chronotropic responses to β -adrenergic receptor stimulation (31). In addition, in these mice, the ability of RyRs to respond to a higher Ca^{2+} load in the SR is reduced, suggesting a link between phosphorylation of RyR2 at Ser²⁸⁰⁸ and sensing of luminal Ca^{2+} concentrations in the SR (32).

There is little information regarding phosphorylation of RyR at Ser²⁸⁰⁹ in sinoatrial node cells. Immunofluorescence analysis indicated that total and phosphorylated RyRs were preferentially distributed beneath the sarcolemma of sinoatrial node cells, in the area where local Ca^{2+} releases were recorded (Fig. 5), whereas ventricular myocytes showed a more uniform distribution of total and phosphorylated RyR (Fig. S6). Raising the concentration of cytosolic Ca^{2+} within the physiological range increased phosphorylation of RyR at Ser²⁸⁰⁹ in permeabilized sinoatrial node cells, but not in ventricular myocytes (Fig. 5E, Fig. S6). Phosphorylation of RyR at Ser²⁸⁰⁹ was reduced in sinoatrial node cells by the PKA inhibitor peptide PKI or the CaMKII inhibitor KN-93, suggesting that this site was phosphorylated by both PKA and CaMKII in these cells. This increased phosphorylation of both RyR and PLB at physiological concentrations of cytosolic Ca^{2+} may promote robust basal Ca^{2+} cycling in sinoatrial node cells.

Both PKA- and CaMKII-dependent phosphorylation events were required for the generation of robust periodic local Ca^{2+} releases in sinoatrial node cells

Suppression of PKA-dependent phosphorylation by PKI resulted in a decrease in the total Ca^{2+} signal mass released by sinoatrial node cells (Fig. 6A) as well as a decrease in the size (Fig. S7A) and periodicity (Fig. 6B) of local Ca^{2+} releases. However the Ca^{2+} content of the SR was unchanged when the cytosolic Ca^{2+} concentration was below 150 nmol/L (Fig. 6C).

Similar to suppression of PKA, suppression of CaMKII-dependent protein phosphorylation by autocalmitide-2 related inhibitory peptide (AIP) in sinoatrial node cells was associated with reduced total Ca^{2+} signal mass (Fig. 7A) and decreased size (Fig. S7D) and periodicity (Fig. 7B) of local Ca^{2+} releases. Similar to PKI, AIP did not change the Ca^{2+} content of the SR when the cytosolic Ca^{2+} concentration was below 150 nmol/L (Fig. 7C). This would be possible only if decreased Ca^{2+} release through RyRs produced by inhibition of either kinase was balanced by reduced Ca^{2+} pumping into SR. Concurrent reductions in the phosphorylation of PLB (23) (Fig. S4C) and phosphorylation of RyR (Fig. 5E) produced by either inhibition of PKA or CaMKII might account for a constant Ca^{2+} content in the SR. At a cytosolic Ca^{2+} concentration of 200 nmol/L, the Ca^{2+} content of the SR was reduced by inhibition of either kinase (Fig. 6C, Fig. 7C), suggesting that less Ca^{2+} was pumped back into the SR by SERCA.

DISCUSSION

Spontaneous beating of sinoatrial nodal pacemaker cells is produced by spontaneous diastolic depolarization, and an ensemble of ion channels and membrane transporters create a “membrane clock” that generates diastolic depolarization and spontaneous action potentials (3, 33). Spontaneous subsarcolemmal local Ca^{2+} releases through RyR occur

during diastolic depolarization and are envisioned to be a “Ca²⁺ clock” (34, 35). In spontaneously firing sinoatrial node cells the “membrane clock” and “Ca²⁺ clock” do not operate in isolation but are mutually entrained through multiple interactions to form a stable, coupled system that drives the automaticity of cardiac pacemaker cells (33–35).

Although Ca²⁺ cycling is essential for the spontaneous activity of cardiac pacemaker cells (1, 4–7, 17, 33–36), the specific features of intracellular Ca²⁺ cycling remain unclear. Here we have demonstrated that sinoatrial node cells generated more robust and rhythmic spontaneous local Ca²⁺ releases through RyRs at the same physiological cytosolic Ca²⁺ concentrations and similar SR Ca²⁺ content than did ventricular myocytes. Sinoatrial node cells and ventricular myocytes had different abundances of proteins involved in Ca²⁺ cycling by the SR [SERCA, PLB, and RyR (Fig. 3, Fig. 5)]. Sinoatrial node cells, but not ventricular myocytes, had Ca²⁺-induced increases of both PKA- and CaMKII-dependent phosphorylation of these proteins.

To sustain a long-lasting, increased Ca²⁺ release through RyRs (Fig. 1) the SR Ca²⁺ stores in sinoatrial node cells must be rapidly replenished by SERCA (7,13). We found that SERCA was more abundant in rabbit sinoatrial node cells than in ventricular myocytes by ~1.5-fold (Fig. 3B). Considering that the amount of PLB in sinoatrial node cells was ~2-fold less than in ventricular myocytes (Fig. 3C), the SERCA/PLB ratio could be at least ~3-fold larger in sinoatrial node cells than in ventricular myocytes. This suggests that there was more uninhibited (not bound to PLB) SERCA in sinoatrial node cells than in ventricular myocytes, which could result in increased Ca²⁺ pumping into the SR which is required to support robust local Ca²⁺ releases. An increase in the cytosolic Ca²⁺ concentration in sinoatrial node cells was associated with increased phosphorylation of PLB at both Ser¹⁶ and Thr¹⁷ (Fig. 4 and Fig. S4, respectively) which would be expected to relieve its inhibition of SERCA-mediated Ca²⁺ pumping. Accordingly, sinoatrial node cells maintained a sufficient SR Ca²⁺ load (Fig. 2), which in the presence of an increased Ca²⁺ release from RyR could be possible only with increased Ca²⁺ reuptake by the SR. Thus, coordinated and synchronized increases in both Ca²⁺ release and reuptake by the SR could sustain large and rhythmic spontaneous local Ca²⁺ releases through RyRs in sinoatrial node cells.

The interplay between Ca²⁺ release through RyRs and reuptake by SERCA is likely to be a major factor that regulates the periodicity of local Ca²⁺ releases. Indeed, each local Ca²⁺ release locally depletes SR Ca²⁺ content and inactivates RyR clusters that generate local Ca²⁺ releases (4). When SR Ca²⁺ is replenished by SERCA and RyRs recover from inactivation, the next spontaneous Ca²⁺ release could occur. Robust, rhythmic local Ca²⁺ releases in pacemaker cells required high basal PKA- and CaMKII- dependent protein phosphorylation because inhibition of either PKA- or CaMKII-dependent phosphorylation resulted in small (Fig. S7A, Fig. S7D), stochastic Ca²⁺ releases that resembled Ca²⁺ sparks in ventricular myocytes (Fig. 6, Fig. 7). Moreover, small and stochastic Ca²⁺ releases in ventricular myocytes also became large and periodic upon exposure to a higher concentration of cytosolic Ca²⁺ (Fig. S2 and S3). Thus, the size of spontaneous Ca²⁺ releases could be a critical factor involved in spontaneous Ca²⁺ release periodicity in both cell types (Fig. S2). When cytosolic Ca²⁺ was increased above 250 nmol/L, the Ca²⁺ content in the SR was increased only in ventricular myocytes (Fig. 2), which led to generation of

large, rhythmic Ca^{2+} wavelets (Fig. S3), whereas the SR Ca^{2+} load (Fig. 2) was decreased and local Ca^{2+} releases were eliminated in sinoatrial node cells (Fig. S3). In permeabilized canine ventricular myocytes exposed to very high cytosolic Ca^{2+} concentrations, RyRs stay continuously open, which promotes a large Ca^{2+} leak and leaves the SR nearly empty of Ca^{2+} thereby suppressing spontaneous Ca^{2+} releases through RyRs (37).

The RyR is a multi-molecular complex that includes regulatory proteins that control Ca^{2+} release from the SR (16). Cytosolic Ca^{2+} activates RyRs in cardiac cells through CICR, and sensitivity of RyRs to cytosolic Ca^{2+} is also regulated by the Ca^{2+} content in the SR (16). However, permeabilized sinoatrial node cells discharge more Ca^{2+} than ventricular myocytes at a similar Ca^{2+} content in the SR, suggesting that other factors account for differences in spontaneous Ca^{2+} releases from RyRs between two cell types. Ca^{2+} -induced phosphorylation of RyRs at physiological cytosolic Ca^{2+} concentrations was enhanced in sinoatrial node cells (Fig. 5), which could be related to the differences in local control mechanisms. In sinoatrial node cells, adenylyl cyclases are constitutively activated by Ca^{2+} (21–22) and the basal concentration of cAMP and basal PKA-dependent phosphorylation are enhanced compared to ventricular myocytes (23). An increase in cytosolic Ca^{2+} concentrations in permeabilized sinoatrial node cells increased cAMP production (Fig. 4A), presumably because of increased activation of adenylyl cyclases, leading to increased PKA-dependent phosphorylation of RyR (Fig. 5E) and, likely, increased sensitivity of RyR to the concentration of luminal Ca^{2+} in the SR. However, it remains to be shown that Ca^{2+} release from RyRs in sinoatrial node cells requires these phosphorylation events. Ca^{2+} -inhibited adenylyl cyclases 5 and 6 are the most abundant adenylyl cyclase isoforms in the ventricle (38), which might partially explain why PKA-dependent phosphorylation of both RyR (Fig. 5E, Fig. S6) and PLB (Fig. 4C) was not observed in ventricular myocytes in response to increased cytosolic Ca^{2+} concentrations.

Physiological concentrations of cytosolic Ca^{2+} in sinoatrial node cells would also be expected to activate CaMKII (24–25), and accordingly, these cells showed increased CaMKII-dependent phosphorylation of PLB (Fig. S4) and RyR (Fig. 5E). Activated CaMKII is located beneath the surface membrane of sinoatrial node cells (39), in the area with highest RyR density (Fig. 5) where local Ca^{2+} releases are generated. In contrast, CaMKII-dependent phosphorylation of PLB (Fig. S4) and RyR (Fig. 5E) in ventricular myocytes was not affected by increased cytosolic Ca^{2+} concentrations, which is consistent with a lack of change in CaMKII-dependent phosphorylation of RyRs in permeabilized canine ventricular myocytes in response to increased cytosolic Ca^{2+} (37).

Study limitations

To compare intrinsic Ca^{2+} cycling at the same physiological cytosolic Ca^{2+} concentration without interference from surface membrane channels we employed saponin-permeabilized sinoatrial node cells and ventricular myocytes. However, there are several caveats regarding permeabilized cells. First, small molecules in the cytoplasm may leak out, which could alter the regulation of intrinsic Ca^{2+} cycling. The phosphorylation response of permeabilized sinoatrial node cells appeared to be intact, suggesting that critical elements of Ca^{2+} cycling in these cells remained intact. Second, it would be an oversimplification to assume that Ca^{2+}

cycling in permeabilized cardiac cells is identical to that within intact cells. Additional modulators of intrinsic Ca^{2+} cycling in cardiac cells include transmembrane ionic channels and membrane-bound regulatory proteins. Different sets of ionic channels and membrane-bound regulatory proteins in intact sinoatrial node cells and ventricular myocytes create additional dissimilarities between Ca^{2+} cycling in these cardiac cells. However, although trade-offs are required for the quantitative comparison between different cell types, we propose that our work can contribute to understanding the differences in intrinsic Ca^{2+} cycling in cardiac pacemaker cells and ventricular myocytes.

The potential clinical relevance of distinct characteristics of Ca^{2+} cycling in cardiac pacemaker cells

Human sinoatrial node dysfunction includes sinus bradycardia and sinus arrest, both of which show increased incidence with aging (2). Implantation of an electronic pacemaker is the common therapy to treat sinoatrial node dysfunction (40); however, it can cause premature death because of electronic failure or chronic infections (41). Although the creation of biological pacemakers is an attractive alternative, attempts to create one have been unsuccessful (42–44). The present study showed that cardiac pacemaker cells rely on Ca^{2+} cycling by the SR to a greater extent than previously assumed. In contrast to current approaches that focus exclusively on ionic channels (42), a robust and functional biological pacemaker design might combine proteins involved in intracellular Ca^{2+} cycling with ionic channels.

MATERIALS AND METHODS

Sinoatrial node cell isolation

Single sinoatrial node cells were isolated from New Zealand White rabbits (Charles River Laboratories, USA) as previously described (6–7). The sinoatrial node region was removed from the heart and cut into ~1.0 mm strips perpendicular to the crista terminalis. The sinoatrial node preparation was washed in Ca^{2+} -free Tyrode solution containing (in mmol/L): 140 NaCl; 5.4 KCl; 0.5 MgCl_2 ; 0.33 NaH_2PO_4 ; 5 HEPES; 5.5 glucose; pH 6.9; and then incubated at $34 \pm 0.5^\circ\text{C}$ for 30 min in Ca^{2+} -free Tyrode solution containing elastase type IV (0.6 mg/ml; Sigma-Aldrich Corp. St. Louis, MO), collagenase type 2 (0.6 mg/ml; Worthington, NJ, USA) and 0.1% bovine serum albumin (Aldrich Corp. St. Louis, MO). Thereafter, the sinoatrial node preparation was washed in modified Kraftbruhe (KB) solution, containing (in mmol/L): 70 potassium glutamate, 30 KCl, 10 KH_2PO_4 , 1 MgCl_2 , 20 taurine, 10 glucose, 0.3 EGTA, and 10 HEPES (titrated to pH 7.4 with KOH), and kept at 4°C for 1 hour in KB solution containing 50 mg/ml polyvinylpyrrolidone (PVP 40, Sigma-Aldrich Corp. St. Louis, MO). Cells were dispersed from the sinoatrial node by gentle pipetting in KB solution and stored at 4°C .

Ventricular myocyte isolation

The rabbit heart was perfused in Langendorff mode for 5 minutes with a nominally Ca^{2+} -free modified Krebs solution containing in mmol/L: 120 NaCl; 5.4 KCl; 1.6 MgSO_4 ; 1.0 NaH_2PO_4 ; 20 NaHCO_3 , at 37°C ; bubbled with 95% O_2 , 5% CO_2 . Perfusion continued for 3–4 min with protease (0.02 mg/ml, Type XIV, Sigma-Aldrich Corp. St. Louis, MO) and

collagenase (1 mg/ml; Type B, 220 to 230 U/mg; Boehringer-Mannheim, Indianapolis, IN; or Type 2, Worthington, Lakewood, NJ, USA), and then for further 10–15 min with 50 $\mu\text{mol/L}$ CaCl_2 added. The ventricles were separated from the heart, chopped into chunks, and placed for a second digestion for 10–15 min in a shaker (60 to 70 rpm) at 37°C, with Tyrode solution containing 100 $\mu\text{mol/L}$ CaCl_2 and 1 mg/ml collagenase. Digestion was quenched by filtering the supernatant for centrifugation at 500 $\times$ g and three washes with a modified Tyrode solution in mmol/L: 137 NaCl; 4.9 KCl; 15 Glucose; 1.2 MgSO_4 ; 1.2 NaH_2PO_4 ; 20 HEPES; (pH=7.4); Ca^{2+} concentrations were successively increased to 250, 500, and 1000 $\mu\text{mol/L}$. Ventricular cells were stored at room temperature in 1 mmol/L Ca^{2+} Tyrode solution.

Cell permeabilization

Intact sinoatrial node or ventricular cells were plated on laminin (20 $\mu\text{g}/1\text{ml}$) in petri dishes (MatTek Culterware) for 20 min to attach. Cells were permeabilized with 0.01% saponin, as previously described (6), in a solution containing in mmol/L: 100 K aspartate; 25 KCl; 10 NaCl; 3 MgATP ; 0.81 MgCl_2 (~1 mM free Mg^{2+}); 20 HEPES; 0.5 EGTA; 10 phosphocreatine and 5U/ml creatine phosphokinase; pH 7.2. After washing out saponin, permeabilization solution was changed to a continuous superfusion with an experimental solution that contained fluo-4 pentapotassium salt (Life Technologies, Grand Island, NY) and Ca^{2+} . The cytosolic free Ca^{2+} at a given total Ca^{2+} , Mg^{2+} , ATP and EGTA concentrations was calculated using a computer program (WinMAXC 2.50, Stanford University, CA).

Confocal imaging of local subsarcolemmal Ca^{2+} releases

Permeabilized sinoatrial node cells or ventricular myocytes loaded with fluo-4 pentapotassium salt were placed on the stage of Zeiss LSM-410 inverted confocal microscope (Carl Zeiss, Inc., Germany). All images of local Ca^{2+} releases in sinoatrial node cells and sparks in ventricular myocytes were recorded in the line-scan mode, with the scan line oriented along the long axis of the cell and processed with IDL software (6.1, Research Systems, Boulder, CO). The bath temperature was maintained at $35\pm0.5^\circ\text{C}$ by temperature controller (Cell Micro Controls, Norfolk, VA). Spontaneous Ca^{2+} releases in permeabilized sinoatrial node cells and ventricular myocytes were analyzed as previously described (6, 23): the amplitude of each spontaneous Ca^{2+} release was calculated as the peak value (F) normalized to minimal fluorescence (F_0), its spatial size was indexed as the full width at half maximum amplitude (FWHM), and its duration characterized as the full duration at half maximum amplitude (FDHM). Parameters of spontaneous Ca^{2+} releases in sinoatrial node cells and ventricular myocytes were different; thus, the number of spontaneous Ca^{2+} releases did not reflect the amount of Ca^{2+} released by each cell type. To avoid this problem, we calculated the total Ca^{2+} signal mass released by each cell type (6, 43). The signal mass of individual Ca^{2+} releases (local Ca^{2+} releases in sinoatrial node cells or sparks in ventricular myocytes) was estimated as follows:

$$M = \text{FWHM} \times \text{FDHM} \times 1/2 \Delta F/F_0 \text{ (where } \Delta F/F_0 = F/F_0 - 1) \quad (\text{I})$$

$[Ca^{2+}]_i$ value in permeabilized cells was calculated using pseudoratio (8):

$$[Ca^{2+}]_i = K_d(F/F_0)/[K_d/[Ca^{2+}]_f + 1 - F/F_0] \quad (II)$$

where K_d is 864 nmol/L (46) and free Ca^{2+} at rest $[Ca^{2+}]_f$ in the “internal” solution in permeabilized cells is known.

The spontaneous Ca^{2+} release amplitudes measured as F/F_0 were converted to changes in Ca^{2+} concentration:

$$\Delta[Ca^{2+}]_i = \text{peak}[Ca^{2+}]_i - \text{rest}[Ca^{2+}]_f \quad (III)$$

where peak $[Ca^{2+}]_i$ was calculated using equation (II) and rest $[Ca^{2+}]_f$ is known.

The total Ca^{2+} signal mass collectively produced by either local Ca^{2+} releases in sinoatrial node cells or sparks in ventricular myocytes was estimated, as previously described (6, 45) by integrating signal masses of all individual Ca^{2+} releases produced by either cell type during 400 msec time-interval and along 100 μm line-scan. In permeabilized sinoatrial node cells local Ca^{2+} releases appear after a specific delay, demonstrating roughly periodic behavior. In contrast, sparks in permeabilized ventricular myocytes are random at low 50–150 nmol/L cytosolic Ca^{2+} , but when free cytosolic Ca^{2+} reaches 200 nmol/L they increase in size and become roughly periodic (Fig. S2, S3). To compare periodicity of spontaneous Ca^{2+} releases in sinoatrial node cells and ventricular myocytes, we employed fast Fourier transform (FFT) (20) using Ca^{2+} waveforms as time domains for FFT. FFT analysis was performed using Clampfit 10.3 (Axon Instruments Inc. Union City, CA) with a rectangular window and the power displayed on a linear scale. To provide similar approach for two different cell types and considering that the spark size in ventricular myocytes is $\sim 2\text{--}3\mu\text{m}$, Ca^{2+} waveforms were calculated from 2–3 μm bands. Although several spots with periodic Ca^{2+} releases could be present in the single sinoatrial node cell, for simplicity, a single spot was enough to consider that the cell had periodic Ca^{2+} releases.

The Ca^{2+} content in the SR in both cell types under control conditions and in the presence of drugs was determined from the peak amplitude of Ca^{2+} transients produced by rapid application of 20 mmol/L caffeine onto permeabilized cells. The change in intracellular $[Ca^{2+}]_i$ on caffeine application (peak $[Ca^{2+}]_i - \text{rest}[Ca^{2+}]_f$ before caffeine) was converted to the change in total SR Ca^{2+} ($Ca^{2+} = [Ca^{2+}]_t - \text{basal}[Ca^{2+}]_i$), as previously described (47), taking into account Ca^{2+} binding by EGTA and Ca^{2+} indicator:

$$[Ca^{2+}]_t = [Ca^{2+}]_i + [EGTA]/(1 + K_{EGTA}/[Ca^{2+}]_i) + [fluo-4]/(1 + K_{fluo}/[Ca^{2+}]_i)$$

where $[EGTA]=0.5$ mmol/L, $[fluo-4]=30$ μM , $K_{fluo}=864$ nmol/L. For this type of experiment, images were taken with the scan line oriented perpendicular to the long axis of the cell to prevent cell shifting during caffeine application.

Western Blot (SERCA)

Heart tissues (atrial, sinoatrial or ventricular) or intact sinoatrial node cells or ventricular myocytes were frozen in liquid nitrogen and subsequently mixed with the ice cold lysis buffer (RIPA: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate 0.1% SDS, 1 mM EDTA) with protease inhibitor cocktail (Sigma-Aldrich Corp. St. Louis, MO). Total protein content was determined using standard commercially available 2D protein Quant kit (GE Healthcare), accuracy of protein quantification was verified using 4–12% Bis-Tris polyacrylamide SDS- PAGE gel (Life Technologies, Grand Island, NY). Total integral of fluorescence intensities for each protein lane was calculated using standard ImageQuant5 software (GE Healthcare, Waukesha, WI). 12 µg of protein from either tissue or cell lysates were mixed with the NuPage sample buffer and DTT; resolved on the 4–12% Bis-Tris polyacrylamide SDS-PAGE gel using standard MOPS running buffer. Proteins were transferred to PVDF membrane and incubated with the primary anti-SERCA mouse monoclonal antibodies (ABR, 1:5000) and subsequently with goat anti-mouse polyclonal-HRP secondary antibodies (Dako North America, Inc., CA). For actin, primary anti-actin goat polyclonal antibodies (Santa Cruz Biotechnology Inc., CA, 1:3000) were used in the same gel, followed by secondary Donkey anti-Goat polyclonal-HRP antibodies (Santa Cruz Biotechnology Inc., CA).

Western blot of PLB in intact or permeabilized sinoatrial node cells and ventricular myocytes

Rabbit sinoatrial node cells and ventricular myocytes were permeablized and incubated at different Ca^{2+} concentrations (0 – 250 nmol/L) for 10 minutes at $35 \pm 0.5^\circ\text{C}$. Subsequently, intact or permeabilized cells were solubilized and extracts were snap-frozen in liquid nitrogen. To dissociate PLB into monomers (6 kD) the samples were boiled at 95°C for 10min. The protein load was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL). PLB proteins were resolved by 7.5% urea/SDS-PAGE gel and transferred (10 µg protein/lane) to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, UK). To detect total PLB, membranes were probed with the total-PLB monoclonal antibody (IgG1, 1:10,000, Badrilla, UK). The detection of site specific PLB phosphorylation at PKA-dependent Ser¹⁶ site was performed using site-specific PS-16 polyclonal antibody (1:10,000, Badrilla, UK) and HRP conjugated anti-rabbit secondary antibody (1:15,000) (Bio-Rad Laboratories, Philadelphia, PA), as previously described (48). PVDF membranes were exposed to the chemiluminescence (ECL, Amersham Pharmacia Biotech, UK) reaction and quantified with a video documentation system (Bio-Rad Laboratories, Philadelphia, PA).

Immunostaining of PLB and RyR in permeabilized rabbit sinoatrial node cells and ventricular myocytes

Rabbit sinoatrial node cells and ventricular myocytes were permeablized and incubated at different Ca^{2+} concentrations (0, 150, 200 or 250 nmol/L) at $35 \pm 0.5^\circ\text{C}$. In the subset of experiments permeabilized sinoatrial node cells were treated with either PKA inhibitor (15 µmol/L PKI) or CaMKII inhibitor (3 µmol/L KN-93 or its inactive analog 3 µmol/L KN-92) for 20 min. The cells were fixed with 4% PFA, treated with 1% Triton and incubated with blocking solution (1×PBS containing 2% BSA + 5% donkey serum + 0.01% NaN_3 + 0.2%

Triton). To assess CaMKII-dependent phosphorylation of Thr¹⁷, permeabilized cells were incubated with an polyclonal antibody specific for phosphorylated Thr¹⁷ (rabbit, 1:200, Badrilla, UK) or total PLB monoclonal antibody (mouse, 1:1000, Badrilla, UK). For RyR2 immunostaining, permeabilized cells were treated with an antibody that recognized total RyR2 (mouse, 1:1000, ABR) and an antibody that recognized RyR2 phosphorylated at Ser²⁸⁰⁹ (rabbit, 1:200, Badrilla, UK) antibody. Secondary Cy5-conjugated anti-mouse IgG antibody (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) were used for either total PLB or total RyR, and secondary Cy3-conjugated anti-rabbit IgG antibody (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA) were used for phosphorylated PLB or RyR immunostaining. Dual confocal images of central sections of sinoatrial node cells and ventricular myocytes were obtained with a Zeiss LSM 510 (Carl Zeiss Inc., Germany). For either RyR or PLB the total amount of protein or fluorophore Cy5 was detected by 633 nm excitation and 650 nm Long-Pass emission, and 543 nm excitation and 565–615 nm Band-Pass emission were used for the detection of phosphorylation or fluorophore Cy3. Considering that cells vary in size and total RyR or PLB protein density might vary from cell to cell (11), the extent of RyR or PLB phosphorylation was estimated as a ratio of phosphorylated to total RyR or PLB protein. Specifically, the average fluorescence density of PLB phosphorylated at Thr¹⁷ in a cell was normalized to total PLB, and fluorescence density of RyR2 phosphorylated at Ser²⁸⁰⁹ in a cell was normalized to the total RyR2 fluorescence density (the nuclear area was excluded in all cells). Only secondary antibodies were applied to the negative control, which displayed negligible fluorescence.

cAMP measurements in permeabilized rabbit sinoatrial node cells

Permeabilized sinoatrial node cells were incubated for 5 min at 35°C in the solution containing 0 or 150–180 nmol/L free cytosolic Ca²⁺. The buffer and sinoatrial node cells were collected and reaction was stopped with ice cold ethanol. Supernatants were adjusted to 65% ethanol and used for cAMP estimation using a LANCE cAMP 384 kit (PerkinElmer, Waltham, MA). Fluorescence intensity was measured on a Victor instrument (PerkinElmer, Waltham, MA) with the LANCE high count 615/665 protocol. Total protein was determined with a BCA assay (Pierce Biotechnology, Rockford, IL). The amount of the cAMP was calculated in pmol/mg protein.

Drugs

The CaMKII inhibitor KN-93, its inactive analog KN-92, the CaMKII inhibitor peptide AIP, and a cell permeable PKA inhibitor peptide Amide 14–22 (PKI) were from EMD4 Biosciences, Calbiochem, Darmstadt, Germany.

Statistics

Data are shown as means ± SEM. Statistical significance was evaluated by t-test and analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Normalized data were analyzed using a one-sample t-test. If heterogeneous random errors have been observed in the data set, a logarithmic transformation of the data was performed before the analysis to stabilize the variance. Logistic regression analysis was used to evaluate statistical significance between fitting curves of periodic Ca²⁺ releases in two different cell types and

in sinoatrial node cells before and after treatment with PKI or AIP. A value of $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Regulation of spontaneous Ca^{2+} releases by cytosolic Ca^{2+} differed in permeabilized sinoatrial node cells (SANC) and ventricular myocytes (VM)
 (A) Representative images and Ca^{2+} waveforms from bands (indicated by arrows) of SANC exposed to different concentrations of cytosolic free Ca^{2+} and (B) FFT of Ca^{2+} waveforms in (A). (C) Representative images and Ca^{2+} waveforms of VM bathed at different cytosolic Ca^{2+} concentrations and (D) FFT of the Ca^{2+} waveforms from bands indicated by the color-matched arrows in (C). (E) Comparison of total Ca^{2+} signal mass released by either SANC (n=9–54 cells for each data point) or VM (n=5–12 cells for each data point) at different cytosolic Ca^{2+} concentrations. (F) Relative number of SANC and VM (% of total) generating periodic spontaneous Ca^{2+} releases. The number of cells with periodic releases is shown as a fraction of the total number of cells studied at each cytosolic Ca^{2+} concentration. Logistic regression analysis demonstrated a significant difference between the curves for SANC and VM ($P < 0.002$). The average FFT frequency of spontaneous Ca^{2+} releases in SANC or VM was ~ 3 Hz at different free cytosolic Ca^{2+} concentrations. * $P < 0.05$.

Fig. 2. The Ca²⁺ content in the SR remained comparable in permeabilized sinoatrial node cells (SANC) and ventricular myocytes (VM) at physiological cytosolic Ca²⁺ concentrations
 (A) Caffeine-induced Ca²⁺ transients in representative SANC and (B) VM at different cytosolic Ca²⁺ concentrations. The initial rapid component of the caffeine-induced Ca²⁺ transient (indicated by arrows) is analyzed. (C) Average total SR Ca²⁺ content in SANC (n=9–31 cells for each data point) and VM (n=6–11 cells for each data point). *P<0.05.

Fig. 3. The SR Ca^{2+} pump SERCA was more abundant and the SERCA inhibitor PLB was less abundant in sinoatrial node cells (SANC) than in ventricular myocytes (VM)

(A) Top, representative western blots of SERCA in right atrium, sinoatrial node (SAN) and ventricular tissues. Bottom, average data (n=3 blots) was normalized and shown as percent of the amount of SERCA protein in ventricular tissue. (B) Representative western blots of SERCA in SANC and VM. Bottom, average data (n=5 blots) are shown as percent of the amount of SERCA protein in VM. (C) Top, representative western blot of total PLB in SANC and VM. Bottom, average data (n=6 blots) are shown as percent of the amount of PLB in VM. * $P < 0.05$.

Fig. 4. Ca²⁺-dependence of PKA-mediated phosphorylation of PLB at Ser¹⁶ differed in permeabilized sinoatrial node cells and ventricular myocytes

(A) An increase in the cytosolic Ca²⁺ concentration was associated with increased cAMP concentration in permeabilized sinoatrial node cells (n=8 rabbits). (B and C) Top, representative western blots of phosphorylated and total PLB in sinoatrial node cells and ventricular myocytes exposed to different cytosolic Ca²⁺ concentrations. Bottom, quantification of PLB phosphorylation in sinoatrial node cells (n=5 blots) and ventricular myocytes (n=4 blots). *P<0.05.

Fig. 5. Phosphorylation of RyR in permeabilized sinoatrial node cells was modulated by the cytosolic Ca^{2+} concentration
 (A and B) Representative confocal images of SANC immunolabeled for total RyR (red) and RyR phosphorylated at Ser²⁸⁰⁹ (green) at the indicated cytosolic Ca^{2+} concentration. (C) and (D) A graph of the pixel-by-pixel fluorescence intensities of total and phosphorylated RyR labeling along an arbitrary line, indicated by a white line in (A) and (B) at the indicated cytosolic Ca^{2+} concentration. The dashed lines in (C) and (D) show the average pixel intensity (over the entire cell) for total RyR (red) and phosphorylated RyR (green). (E) Relative changes in the phosphorylation of RyR at Ser²⁸⁰⁹ normalized to total RyR in SANC

exposed to different cytosolic Ca^{2+} concentrations (n=49–80 cells for each data point) and VM (n=80–81 cells for each data point). *P<0.05.

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Fig. 6. Inhibition of PKA-dependent protein phosphorylation suppressed spontaneous, periodic, local Ca^{2+} releases in permeabilized sinoatrial node cells (SANC)

(A) left, Confocal line-scan images of a representative SANC bathed in 200 nmol/L cytosolic Ca^{2+} before (top) and after (bottom) superfusion with PKI. Right, total Ca^{2+} signal mass released by SANC before and after superfusion with PKI ($n=5-12$ cells for each data point). (B) Left, FFT of Ca^{2+} oscillations (from bands indicated by arrows) in (A), before and after PKI. Right, Relative number of SANC that generated periodic spontaneous Ca^{2+} releases in control and after PKI; logistic regression analysis demonstrated a significant difference between curves ($P<0.0001$). (C) Left, representative images and caffeine-induced

Ca²⁺ transients in SANC in control conditions and after treatment with PKI. Right, average data of the total Ca²⁺ content in the SR before and after PKI treatment. n=3–9 cells for each data point. *P<0.05.

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Fig. 7. Inhibition of CaMKII suppressed spontaneous, periodic, local Ca^{2+} releases in permeabilized sinoatrial node cells (SANC)

(A) Left, Confocal linescan images of a representative SANC bathed in 200 nmol/L cytosolic Ca^{2+} before (top) and after (bottom) superfusion with AIP. Right, AIP treatment resulted in decreased total Ca^{2+} signal mass released by SANC. $n=4-8$ cells for each data point. (B) Left, FFT of Ca^{2+} oscillations (from bands indicated by arrows) in (A), before and after AIP treatment. Right, relative number of SANC that generated periodic spontaneous Ca^{2+} releases in control conditions and after AIP treatment. Logistic regression analysis demonstrated a significant difference between the two curves ($P < 0.002$). (C) Left, representative images and caffeine-induced Ca^{2+} transients in control conditions and after treatment with AIP. Right, average data of the total SR Ca^{2+} content before and after AIP treatment ($n=3-13$ cells for each data point). $*P < 0.05$.