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Local Control of Ca^{2+} -induced Ca^{2+} release in mouse sinoatrial node cells

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Abstract

Emerging evidence from large animal models implicates Ca^{2+} regulation, particularly intracellular sarcoplasmic reticulum (SR) Ca^{2+} release, as essential for sinoatrial node (SAN) automaticity. However, despite the apparent importance of SR Ca^{2+} release to SAN cell function it is uncertain how SR Ca^{2+} release is controlled in SAN cells from mouse. Understanding mouse SAN SR Ca^{2+} release mechanism will allow improved understanding of results in studies on SAN from genetic mouse models of Ca^{2+} homeostatic proteins. Here we investigated the functional relationship between sarcolemmal Ca^{2+} influx and SR Ca^{2+} release at the level of single SAN cell, using simultaneous patch-clamp current recording and high resolution confocal Ca^{2+} imaging techniques. In mouse SAN cells, both Ca^{2+} channel currents and triggered SR Ca^{2+} transients displayed bell-shaped, graded function with the membrane potential. Moreover, the gain function for Ca^{2+} -induced Ca^{2+} release (CICR) displayed a monotonically decreasing function with strong voltage-dependence, consistent with a 'local control' mechanism for CICR. In addition, we observed numerous discrete Ca^{2+} sparks at the voltage range of diastolic depolarization, in sharp contrast to the much lower frequency of sparks observed at resting potentials. We concluded that the 'local control' mechanism of CICR is responsible for both local Ca^{2+} release during diastolic depolarization and the synchronized Ca^{2+} transients observed during action potential in SAN cells.

Keywords

sinoatrial node; automaticity; diastolic depolarization; Ca^{2+} sparks; local control

Introduction

Normal cardiac automaticity is regulated by a small group of specialized cardiac myocytes in the sinoatrial node (SAN). These autonomous pacemaking cells are characterized by spontaneous electrical depolarization during late diastole (so called phase 4) [1,2]. Increasing evidence supports a concept that intracellular calcium ($[\text{Ca}^{2+}]_i$) plays a critical role in regulation

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of SAN automaticity [3,4]. Moreover, recent evidence from large animal models suggests that the control of SAN $[Ca^{2+}]_i$ may be modulated by the coordinated relationship between voltage-gated sarcolemmal calcium channels and sarcoplasmic reticulum (SR) calcium-release channels (RyRs) [5,6]. Despite recent reports detailing the importance of SR calcium in SAN automaticity, the understanding of the origin and cellular mechanisms regulating SR Ca^{2+} release of SAN cells is incomplete and has not been described in the mouse.

We hypothesize that SAN cells have evolved “local control” mechanisms to regulate cytosolic Ca^{2+} and SAN automaticity. We know most about local control of intracellular Ca^{2+} release from studies in ventricular myocytes [7–9]. In ventricular myocytes, RyR Ca^{2+} release channels on SR membranes are tightly juxtaposed (~10–12 nm) with L-type Ca^{2+} channels resident in transverse-tubular (T-tubule) sarcolemmal membrane invaginations [10]. During membrane depolarization, inward Ca^{2+} current ($I_{Ca,L}$) through L-type Ca^{2+} channels activates SR RyRs resulting in much greater increase in cytosolic Ca^{2+} . This process, termed Ca^{2+} -induced Ca^{2+} release (CICR) [11], is ‘locally controlled’, and graded with high amplification [7]. Moreover, this process is highly synchronized among tens of thousands of highly organized calcium release units within the myocyte. The synchrony of SR Ca^{2+} release is made possible by the widely distributed and regularly arrayed T-tubules that allow instantaneous electrical excitation and synchronous triggering of Ca^{2+} release throughout the entire cytoplasm [12].

In contrast to ventricular myocytes, SAN cells lack T-tubules [13]. The membrane associated molecules (e.g. channels, transporters) responsible for depolarizing and repolarizing currents in SAN cells are in many cases different from their counterparts in ventricular myocytes. Finally, the primary goal of the SAN cell is not contraction. Rather, the SAN cell has clearly evolved to regulate automaticity. Despite these significant differences, preliminary evidence supports a close functional relationship between sarcolemmal Ca^{2+} channels and SR Ca^{2+} release in the metazoan SAN from rabbit SAN and cat latent pacemaker cells [5,6]. In this study, we attempt to understand the Ca^{2+} regulatory mechanism of SAN cells from mouse.

We investigated local Ca^{2+} control mechanism at the level of single mouse SAN cell. Here, we demonstrate that SAN SR Ca^{2+} release in the subsarcolemmal space is tightly controlled by sarcolemmal Ca^{2+} channels, similar to that of ventricular myocytes. Our findings support a model where a local control mechanism for CICR is responsible for both local Ca^{2+} release during ‘phase 4’ late diastolic depolarization and global cytosolic Ca^{2+} transients during the action potential upstroke in SAN cells. Secondly, local SR Ca^{2+} release events observed during diastolic depolarization stage are mostly attributed to membrane potential dependent - local control CICR, rather than spontaneous Ca^{2+} oscillation. Thus, our data suggest that while maintaining clearly unique cellular roles (automaticity vs. contraction) and striking molecular and structural differences, the SAN cell and ventricular myocyte have adapted unexpectedly similar mechanisms for intracellular Ca^{2+} regulation.

Materials and Methods

Mouse SAN cell isolation

Isolation of single SAN cells from control mice (CD1, from Charles River Laboratory, USA) was performed as modified from Mangoni and Nargeot [14]. Mice were administered an intraperitoneal injection of heparin (1000 IU/ml) and avertin (20 μ l/g). The heart was excised and placed into Tyrode’s solution (35°C), consisting of (mM) 140 NaCl, 5.0 HEPES, 5.5 Glucose, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, with 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 was cut into smaller pieces, which were transferred and rinsed in a ‘low Ca^{2+} ’ solution containing (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. SAN tissue pieces were digested in 5 ml of 'low Ca^{2+} ' solution containing collagenase type I, elastase (Worthington), and protease type XIV (Sigma) for 20–30 min. Then the tissue was transferred to 10 ml of Kraft-Bruhe medium containing (mM) 100 potassium glutamate, 5.0 HEPES, 20 Glucose, 25 KCl, 10 potassium aspartate, 2.0 MgSO_4 , 10 KH_2PO_4 , 20 taurine, 5 creatine, 0.5 EGTA, and 1 mg/ml BSA, with pH adjusted to 7.2 with KOH. The tissue was agitated using a glass pipette for 10 min. The cells were stored at 4°C and studied within seven hours.

SAN cells were placed in Tyrode's solution at 36 ± 0.5 °C. SAN cells were identified by their characteristic morphology (spindle or spider shape) and rhythmic spontaneous activity. SAN cells were identified electrophysiologically by typical spontaneous action potentials with slow depolarizing phase 4 and the hyperpolarization-activated current I_f (Supplemental Figure S1). The average cell capacitance of the cells used in this study is 36.3 ± 2.9 pF ($n=14$).

Spontaneous action potentials (APs) were recorded using the perforated (amphotericin B) patch-clamp technique on single SAN cells at 36 ± 0.5 °C in Tyrode's solution with the pipette filled with (mM) 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 CaCl_2 , 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, amphotericin B 240 $\mu\text{g/ml}$, with pH adjusted to 7.2 with KOH. SAN cells with stable APs were included in the experiments.

Confocal Ca^{2+} imaging in SAN cells

Single isolated SAN cells were loaded with an optimized loading protocol to avoid excessive Ca^{2+} buffering by Ca^{2+} indicator (5 μM Fluo-4 AM at 10 minutes, and then rested for 20 minutes in normal Tyrode's solution for de-esterization). After placement on a recording chamber, the cells were perfused in normal Tyrode's solution at $36^\circ\text{C} \pm 0.5$ (Temperature Controller, TC2BIP, Cell MicroControls, VA). Spindle or spider-shaped, actively spontaneous beating cells were chosen for experiments. Confocal Ca^{2+} imaging (LSM510, Carl Zeiss MicroImaging) was performed in line scan mode at a speed of 1.92 ms per line. The scan lines were scanned along the cell edge, because Ca^{2+} release occurs mostly in the SAN cell periphery (Supplemental Figure S2) [5].

There are four series of experiments performed with the confocal system [15–17]. 1) *Spontaneous Ca^{2+} transients recorded with confocal imaging alone.* 2) *Simultaneous voltage clamp recording of Ca^{2+} current and confocal Ca^{2+} imaging.* These experiments were performed either in ruptured patch clamp or in perforated patch clamp configuration. Pipette resistance ranges between 4–5 M Ω . Ca^{2+} indicators, Fluo-4 pentapotassium salt (or AM form) was either dialyzed into the cells through ruptured patch or preloaded in Tyrode solution (Fluo-4 AM) before electrophysiology. The pipette solution contains (in mM) CsCl 110, TEACl 20, NaCl 10, GTP- Na_2 0.4, Mg-ATP 5, HEPES 10, MgCl_2 1, pH adjusted to 7.2 with CsOH. The bath solution contains (mM): NaCl 140, KCl 5, MgCl_2 0.5, CaCl_2 2, NaH_2PO_4 0.33, glucose 10 and HEPES 10, pH adjusted to 7.4 with NaOH. After establishment of ruptured / perforated patch, the cells were switched to recording solution containing (mM) NaCl 140, CsCl 10, MgCl_2 0.5, CaCl_2 2, NaH_2PO_4 0.33, glucose 10, HEPES 10 and TTX 0.02, pH 7.4. Simultaneous recording of Ca^{2+} currents and confocal Ca^{2+} imaging was executed within 5 minutes. **The voltage protocol** for Ca^{2+} currents / Ca^{2+} imaging was as follows (See also Figure 4A). The cell was clamped to a holding potential of -60 mV, and voltage command was running at 10 seconds interval. Starting with a ramp depolarization to -40 mV from -60 mV (500 ms) and then a holding command at -40 mV for 100 ms, the cell was then depolarized to different test voltages ranging from -40 to $+50$ mV in 10 mV increments (300 ms). After the test pulses, the cell was repolarized to -60 mV. -60 mV was chosen as the holding potential for all experiments as the maximal diastolic potential of mouse SAN cells is approximately -60 mV [2]. The ramp depolarization to -40 mV from -60 mV was utilized to mimic the range of phase 4 cell membrane potentials. A prolonged ramp depolarization (500 ms) was used to

amplify and distinguish the effects of different membrane components in triggering local SR Ca^{2+} release at phase 4. 3) “ Ca^{2+} spike” measurement [16]. Some studies were performed in ruptured patch-clamp mode, since specific pipette/intracellular solution was required for this assay. In addition to the constituents described above for the pipette filling solution, 4 mM EGTA and 2 mM CaCl_2 were added to the pipette solution with a final free Ca^{2+} concentration of 150 nM [16]. After achievement of whole-cell mode, simultaneous confocal Ca^{2+} imaging and voltage clamp current recording were performed as described above. This assay has been used to directly measure SR release function in ventricular myocytes [16]. In this study, we adapted this method to further examine the nature of local Ca^{2+} release events during diastolic depolarization. 4) *Simultaneous current clamp recording of SAN action potential and confocal Ca^{2+} imaging*. These experiments were performed under current-clamp configuration, as we have reported previously [18], with perforated patch-clamp technique on Fluo-4 AM preloaded SAN cells. Electrode pipette resistance ranges between 3 and 5 M Ω . Perforated patch was achieved by using Amphotericin B (240 μM) in pipette solution containing (in mM) K^+ -Aspartate 130, NaCl 10, GTP- Na^{2+} 0.1, Mg-ATP 2, HEPES 10, CaCl_2 0.04 (pH adjusted to 7.2 with KOH). The inclusion of 0.04 mM CaCl_2 in pipette solution is to guarantee a perforated patch rather than ruptured patch (Ca^{2+} ion can't go through the perforated patch. Cells will die because of the high Ca^{2+} if cell membrane is broken through). The bath solution contains (mM): NaCl 140, KCl 5, MgCl_2 1, CaCl_2 2, NaH_2PO_4 0.33, glucose 10 and HEPES 10, pH 7.4 adjusted with NaOH. A successful perforation of the membrane patch will be achieved when the series resistance stabilizes (normally reaches to 20–30 m Ω). Patch clamp system and confocal microscope will be set to simultaneously monitor/acquire action potentials and corresponding Ca^{2+} signals without giving any current stimulus to the cells. The synchronization between confocal Ca^{2+} signals and electrical activities was achieved by sending a trigger signal from confocal imaging system to Digidata 1440A controlled by pClamp 10 (Molecular Devices Inc., USA) right before starting image acquisition.

Data Analysis and Statistics

All electrophysiology data were analyzed offline with clampfit (Version 10, Molecular Devices, Inc.). Analysis of Ca^{2+} transients and sparks was performed offline with home-compiled routines in IDL program (Research System Inc., Colorado), as described previously [19]. Paired or unpaired Student's *t* test was used to test the statistical significance between groups, as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Mouse SAN cells display stable and graded calcium-induced calcium release

Despite the fact that mice represent the primary animal model to investigate the molecular mechanisms underlying human cardiovascular disease, little is known regarding the cellular pathways governing mouse SAN Ca^{2+} release. Therefore we first examined SR Ca^{2+} release in freshly isolated mouse SAN cells. Normally, Mouse SAN cells displayed spontaneous, rhythmic Ca^{2+} transients (Figure 1). Local Ca^{2+} release events (or Ca^{2+} sparks) were frequent, and primarily occurred during the late phase of diastolic depolarization preceding the initiation of the cell action potential (Figure 1 and Figure 2). Consistent with previous observations from larger animal models, we observed many SR Ca^{2+} release events near the cell periphery (Figure S2) [5]. Ca^{2+} transients measured within the subsarcolemmal space were highly synchronized. Moreover, the Ca^{2+} transients remained stable from beat to beat under either spontaneous beating or current clamped conditions (Figure 1–Figure 2). These findings suggest that SAN subsarcolemmal SR Ca^{2+} release is controlled, at least partially, by a mechanism similar to ventricular myocyte CICR.

We next examined the functional relationship between sarcolemmal Ca^{2+} influx and SR Ca^{2+} release in mouse SAN cells by determining the relationship between membrane potential, Ca^{2+} influx through the L-type Ca^{2+} channel ($I_{\text{Ca,L}}$), and SR Ca^{2+} release. Our new data suggest that plasma membrane $I_{\text{Ca,L}}$ triggers graded SR Ca^{2+} release in SAN cells. Specifically, when test voltages were negative (e.g. -30 mV), $I_{\text{Ca,L}}$ was small (Figure 3D). At -30 mV, the corresponding SAN SR Ca^{2+} release was scattered and asynchronous (Figure 3B). However, as $I_{\text{Ca,L}}$ increased (in response to a more positive voltage command pulses, e.g. 0 and $+10$ mV), the corresponding SAN cell Ca^{2+} transients became elevated and synchronized (Figure 3B–D). Additionally, as $I_{\text{Ca,L}}$ decreased (at a voltage of $+30$ mV or greater), we observed a gradual decrease of SR Ca^{2+} release that corresponded to a reduction in inward $I_{\text{Ca,L}}$. Finally, a gradual increase of the Ca^{2+} transient was observed upon repolarization of the SAN membrane potential, due to the increased $I_{\text{Ca,L}}$ in response to step-increased driving forces (Figure 3B–D). Taken together, these data suggest that both depolarization- and repolarization-associated $I_{\text{Ca,L}}$ triggers graded SR Ca^{2+} release in mouse SAN cells. The steep rising phase of the SAN Ca^{2+} transient (as short as 35 ms, Figure 3C, Figure 4D), also suggests that the plasma membrane $I_{\text{Ca,L}}$ and SAN SR Ca^{2+} release are functionally coupled within a restricted subsarcolemmal microdomain.

Analysis of SR Ca^{2+} release over an entire range of test pulses revealed that SAN myocytes display graded Ca^{2+} release mechanisms. Specifically, both the trigger signal ($I_{\text{Ca,L}}$ current density) and the corresponding SR Ca^{2+} release event exhibited a classic “bell shaped” dependence on membrane potential (Figure 4A–B, $n=8$) [20]. The SAN gain function, measured as a ratio of the peak amplitude of SR Ca^{2+} transient to its corresponding Ca^{2+} trigger signal, also exhibited strong voltage-dependence (Figure 4C). This variable, monotonically decreasing gain function observed in SAN cells was strikingly similar to that observed in ventricular myocytes [8, 21, 22]. The higher gain function at low voltages comparing to high positive voltages is probably associated with the larger unitary current, lower open probability and fewer redundant Ca^{2+} channel openings of single L-type Ca^{2+} channels at the low voltages, as suggested in a study on ventricular myocytes [22]. Furthermore, the rising kinetics of SAN Ca^{2+} transients (an index of synchrony of Ca^{2+} release) were virtually identical to observations from mouse ventricular cardiomyocytes (Figure 4D). These data strongly support the concept that SAN cell SR Ca^{2+} release is tightly controlled by sarcolemmal Ca^{2+} channels through a “local control” mechanism, similar to CICR in ventricular myocytes [7], or CICR in close proximity to the subsarcolemma in atrial myocytes [23, 24].

Triggered SAN Ca^{2+} sparks occur during phase 4 depolarization

Local SR Ca^{2+} release during the phase 4 diastolic depolarization stage is believed to play a crucial role for SAN cell spontaneous diastolic depolarization. We next sought to better understand the nature of these local Ca^{2+} release events. During voltage-clamped test pulses, we observed synchronized Ca^{2+} transients in subsarcolemmal space, particularly at more positive voltages (Figure 3, e.g. 0 mV). However, at more negative voltages, (e.g., -30 mV), Ca^{2+} sparks were discrete and scattered across the depolarization period. This finding, likely due to non-synchronized opening of L-type Ca^{2+} channels at low voltages, is similar to what we have previously observed in ventricular myocytes [16,21]. The scattered Ca^{2+} spark pattern disappeared as the test pulses stepped to more positive voltages (Figure 3B). Interestingly, numerous discrete Ca^{2+} sparks were consistently observed during ramp depolarization (from -60 mV to -40 mV). In contrast, we did not observe frequent sparks during ramp depolarization in ventricular myocytes using the same experimental protocol (data not shown). The frequency of Ca^{2+} sparks during this ramp period was significantly higher than spark events at the holding potential of -60 mV (63.3 ± 1.59 sparks per sec $\cdot 100 \mu\text{m}$, $n=4$ cells at ramp and 10.9 ± 3.4 sparks per sec $\cdot 100 \mu\text{m}$, $n=4$ cells at -60 mV, respectively, $p<0.001$, Figure 5A). Spatial averaging of all Ca^{2+} signals during ramp depolarization (10 images/cell, from -60 to -40 mV) revealed

a monotonic increase in Ca^{2+} fluorescence (Figure 5B). Furthermore, we calculated that mouse SAN cells begin to trigger sparks at ~ -53 mV (-53.2 ± 0.37 mV, $n=8$ cells). Prior to this potential, mouse SAN cells were typically quiescent, with occasional sparks, at a frequency similar to that at a holding potential of -60 mV (see Figure 3B). Together, these observations indicate that the reduced frequency of Ca^{2+} sparks at -60 mV is not likely due to a decrease of SR Ca^{2+} content following previous Ca^{2+} release from the same region. Rather, these data suggest that the occurrence of SAN sparks at ramp voltages is dependent on the specific membrane potential. Thus local Ca^{2+} sparks during diastolic depolarization potential range are likely triggered, rather than spontaneous events in the mouse SAN cell.

Characteristics of elementary local Ca^{2+} release events in mouse SAN cells

Local SR Ca^{2+} release plays an important role in SAN pacemaking activities. However, the properties of the elementary SR Ca^{2+} release events [25] of mouse SAN cells are unknown. We therefore measured the unitary properties of mouse SAN Ca^{2+} sparks during ramp depolarization and at a holding potential of -60 mV. Our results revealed that the unitary properties, including spark amplitude, spatial width, and duration were the same between spontaneous sparks, and sparks triggered by sarcolemmal Ca^{2+} channels (Figure 6). On average, SAN Ca^{2+} sparks were 2.67 ± 0.07 in amplitude (F/F_0), 25.33 ± 0.53 ms long (FDHM) and 2.40 ± 0.06 μm wide (FWHM) during ramp depolarization ($n=445$) and 2.53 ± 0.13 in amplitude, 27.44 ± 1.24 ms long and 2.33 ± 0.11 μm wide at -60 mV ($n=205$; $p>0.05$ for all these three parameters at -60 mV vs. sparks during ramp). Moreover, the histogram distributions of spark parameters were also similar. These results suggest that spontaneous and triggered sparks measured in the subsarcolemmal space arise from the same population of Ca^{2+} release units (RyR clusters) that are physically juxtaposed with the sarcolemmal membrane of SAN cells.

High frequency of local Ca^{2+} release events during phase 4 was not diminished by a high concentration of EGTA

To further investigate the nature of localized Ca^{2+} release during diastolic depolarization, we applied the “ Ca^{2+} spike” assay in SAN cells using a high concentration of EGTA (4 mM) in the pipette solution (see methods). The high concentration of EGTA was shown previously not to affect the local cross-signaling between L-type Ca^{2+} channels and RyRs in ventricular myocytes, but could prohibit propagated or secondary CICR [15,26]. In the presence of EGTA buffer, these local Ca^{2+} release events, called Ca^{2+} spikes, are smaller in size and shorter in duration compared to control conditions (Figure 7). The global Ca^{2+} transient in response to 0 mV step depolarization (from -40 mV) also displays a spike like change with quick rise and decay phases (half time is approximately 20 ms) (Figure 7). Interestingly, in SAN cells the frequent occurrence of local Ca^{2+} release during diastolic depolarization (comparing to that at -60 mV holding potential) was not altered by EGTA buffering (Figure 7). Similar findings were observed in 4 other SAN cells. These data further support our notion of local control CICR in SAN cells.

Discussion

Nearly two decades ago, Rubenstein and Lipsius provided the first evidence that SR Ca^{2+} release contributes significantly to diastolic depolarization, particularly to the late phase of diastolic depolarization in cat atrial latent pacemaker cells [27]. Since then other investigators have supported the critical role of SR Ca^{2+} release in modulating the diastolic depolarization, and thus SAN automaticity in rabbit, guinea pig, mouse and even frog [5,28–33]. Our present work provides the first evidence that definitively identifies the presence of local calcium control mechanisms in mouse SAN cells, and raises exciting new questions regarding the underlying structural and molecular mechanisms that account for the functional relationship between SAN plasma membrane and SR membrane components.

Local Control Mechanism of SR Ca^{2+} release in mouse primary SAN cells

Increasing evidence has suggested an important role of SR Ca^{2+} release in regulating SAN pacemaking activities. Confocal Ca^{2+} imaging with high spatial and temporal resolution has indicated that SR Ca^{2+} release in the subsarcolemmal space is synchronized by and associated with the action potential upstroke, suggesting highly coordinated SR Ca^{2+} release among different clusters of Ca^{2+} release units during the SAN action potential. However, the underlying cellular and molecular mechanism is unclear. SAN cells are specialized class of cardiac cells, that differ both structurally and molecularly from ventricular myocytes [2,34]. For example, SAN cells display spontaneous diastolic depolarization, lack T-tubule membrane structures, unlike healthy ventricular myocytes. While SAN cells share some similarities in Ca^{2+} handling proteins (Cav1.2, RyR2, NCX1, and SERCA2), the SAN cell expresses a number of plasma membrane and SR ion channels not found in the ventricle (Cav1.3, Cav3.1, Cav3.2, RyR3) [2]. In ventricular myocytes, it is well accepted that CICR is a local control process in the nanoscale junctional space between Cav1.2 (on T-tubule) and RyR2 (on SR membrane) [7,10]. It was yet not clear how SR Ca^{2+} release is controlled in SAN cells, or whether SAN cells utilize the same mechanism for controlling SR Ca^{2+} release (e.g., “local control of CICR”).

Our data (Figure 3–Figure 5, Figure 7) suggest that a similar local control CICR mechanism exists in SAN cells, particularly in the periphery or subsarcolemmal space. Likely as a consequence of the lack of T-tubules, we found the SR Ca^{2+} release sites are primarily located close to the cell surface (Figure S2). These data are consistent with RyR2 distribution identified with immunofluorescence in mouse SAN cells by our group [35] and in rabbit SAN cells from other groups [36,37]. Line-scan confocal Ca^{2+} images showed that action potential triggered SR Ca^{2+} releases are highly synchronized along the periphery of SAN cells, suggesting highly coordinated recruitment of SR Ca^{2+} release from different release units/sites during action potential (post diastolic depolarization). The bell shaped voltage dependence of $I_{\text{Ca,L}}$ and SR Ca^{2+} transients in SAN cells is similar to that of ventricular myocytes, as is the monotonically decreasing gain function [8]. These data strongly suggest that sarcolemmal L-type Ca^{2+} channels and RyRs on SR membrane function as a Ca^{2+} release unit operated by a local control mechanism, a process which is insensitive to a high concentration of the slow Ca^{2+} chelator EGTA (Figure 7). This local control mechanism is also well supported by the ultrastructure of subsarcolemmal SR cisternae observed in rat SAN cells [13] and in cat subsidiary pacemaker cells [38], although other studies reported no SR components in SAN cells or so-called P-cells [39,40].

Controlled local SR Ca^{2+} release during phase 4 depolarization in SAN cells

The discrete Ca^{2+} sparks observed at the voltage range between ~ -53 mV and -40 mV during ramp depolarization (from -60 mV to -40 mV) and their significantly higher frequency than that at -60 mV suggest that these Ca^{2+} sparks are membrane potential dependent and likely triggered by Ca^{2+} channels that are activated during this specific voltage range. Since these local Ca^{2+} sparks were from the same subsarcolemmal region as these synchronized Ca^{2+} transient signals, and were dependent on membrane depolarization, our studies suggest that a local control CICR mechanism underlies local Ca^{2+} release during phase 4 diastolic depolarization. Another important finding of this study is that the frequent occurrence of local Ca^{2+} release events during ramp depolarization (comparing to that at -60 mV holding potential) was not altered by the slow Ca^{2+} chelator, EGTA. This result is consistent with local control model of CICR in SAN cells. Interestingly, our findings in mouse SAN cells were similar to a report on cat latent pacemaker cells by Huser and colleagues [6], in which they showed that Ca^{2+} influx via low-voltage activated Ca^{2+} channels stimulates local subsarcolemmal SR Ca^{2+} sparks, indicating that latent and primary pacemaker cells may utilize the same mechanism for regulating SR Ca^{2+} release and tuning heart rhythm. Although SR loading status

is a critical factor in determining both the coupling efficiency between L-type Ca^{2+} channels and RyRs, and also the frequency of spontaneous Ca^{2+} sparks [20,41]. It seems unlikely that a sharp increase in SR Ca^{2+} content during ramp depolarization, especially at voltages negative than -50 mV, leads to the sudden increase of local Ca^{2+} spark firings. We conclude that local control CICR mechanism is critical for both localized Ca^{2+} sparks during diastolic depolarization phase and synchronized Ca^{2+} transients during SAN action potential (in the subsarcolemmal space), and plays important role in spontaneous diastolic depolarization.

Possible molecular determinants responsible for triggering local Ca^{2+} sparks during phase 4 depolarization

Unlike ventricular cardiomyocytes, SAN cells display two distinct families of voltage-gated calcium channels (L-type and T-type). Moreover, SAN cells express at least two subtypes of each calcium channel family (Cav1.2 and Cav1.3; Cav3.1 and Cav3.2) [2]. Relative to Cav1.2 (primary high voltage-activated calcium channel in ventricular cardiomyocytes, activated at -40 mV or more positive voltages), Cav1.3, Cav3.1, and Cav3.2 are low-voltage activated calcium channels [42,43]. We predict that Cav1.2 Ca^{2+} channels are less likely a contributing factor to the ramp depolarization-associated local SR Ca^{2+} release under normal conditions, although there is no doubt that Cav1.2 Ca^{2+} channels are critical for SAN pacemaking function [44,45]. The low-voltage calcium channels are believed to be the primary pathway for calcium influx during phase 4 diastolic depolarization. Cav1.3 is activated at the diastolic depolarization range (from -60 mV to -40 mV). The steady state activation voltage for Cav3.1 and Cav3.2 channels are more negative than Cav1.3 Ca^{2+} channels [2]. The peak amplitude of Cav1.3 channel and Cav3.1 channel current is at similar size (~ 5 pA/pF, peak voltage = -20 mV and -40 mV, respectively), greater than that of Cav1.2 channel current (~ 2 pA/pF at 0 mV) [43, 46]. The current amplitude of Cav3.2 channels is yet unknown in SAN cells. A report showed that Cav3.1 expression is higher than that of Cav3.2 in adult SAN [47]. Importantly, mice homozygous for a null mutation in Cav1.3 or Cav3.1 both displayed sinus bradycardia and increased dispersion of beating (R-R) intervals, but not the mice with Cav3.2 inactivation [43,46,48–50]. Taken together, we postulate that Cav1.3 and Cav3.1 Ca^{2+} channels are the critical pathways providing trigger Ca^{2+} for spark activation during diastolic depolarization.

The exact mechanism underlying SAN automaticity is now under intense debate [51]. It has been long believed that hyperpolarization-activated cyclic nucleotide-gated nonselective cation current (I_f), namely pacemaker current or funny current, is the primary or possibly exclusive, current responsible for phase 4 diastolic depolarization [52,53]. However, functional evidence from I_f block experiments (Cs^+) and recent genetic approaches indicate that I_f does participate in the generation of pacemaker activity, but may not be an absolute prerequisite for automaticity [2]. Since 2000, this mechanism was challenged by Lakatta and his colleagues. They have published a series of work with both experimental and theoretical simulation data and have argued strongly that, instead of I_f , spontaneous local SR Ca^{2+} release is the most important determinant in rabbit SAN automaticity [3]. In our point of view, it's likely that I_f and spontaneous local Ca^{2+} release driven inward NCX current may provide the initial driving force to partially depolarize SAN cells from maximal diastolic potential to the threshold voltage (e.g., ~ -53 mV in mouse SAN) for activating the local control mechanism described above.

Future work with genetically modified mice will be critical to define the molecular trigger(s) underlying SAN local Ca^{2+} sparks during diastolic depolarization. Moreover, additional studies on mouse SAN ultrastructure using immuno-electron microscopy will be important to match the growing literature of functional SAN data with subsarcolemmal structure. Understanding the critical importance of CICR in cardiac pacemaker function is a necessary first step to dissecting Ca^{2+} -based pathways in cardiac pacemaker cells and for developing new therapies for preventing SAN failure.

Comparison of with previous studies and the significance of the present study

Early studies from Lipsius and his colleagues have provided important information on nickel sensitive, low voltage activated Ca^{2+} sparks during diastolic depolarization [6]. However this study was performed on cat latent atrial pacemaker cells, localized in specific regions of the right atrium outside of the SAN. Therefore, the results from this study should not depreciate the novelty of the present work. It would be also necessary to examine this local control mechanism in SAN cells of large animal models, which display much slower heart rates. Our preliminary data from canine SAN cells did show similar voltage dependent local Ca^{2+} sparks during ramp depolarization, but further careful experiments are warranted. Recently, Dr. Lakatta's group has made significant efforts on SR Ca^{2+} release and sinus node pacemaking function in rabbit SAN cells. Their key finding is that rhythmic spontaneous local Ca^{2+} release is the center initiator in phase 4 diastolic depolarization in SAN cells [3,51]. However, the data from our present work strongly suggest that the local Ca^{2+} release during diastolic depolarization stage is primarily membrane potential dependent, by CICR triggering from sarcolemmal Ca^{2+} channels. Therefore, our current study suggests that an integrated CICR mechanism, rather than spontaneous SR Ca^{2+} release, directs the Ca^{2+} -dependent component of SAN automaticity. It is critical that we understand the baseline mechanisms for Ca^{2+} regulation in mouse SAN cells before additional molecular studies are undertaken to dissect molecular cause/effect relationship using the rich variety of available genetic mouse models, which will inform and complement information derived from large animal studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Localized Ca^{2+} sparks and rhythmic Ca^{2+} transients in a spontaneously beating mouse SAN cell

A, Spontaneous rhythmic Ca^{2+} transients (loaded with Fluo-4 AM) recorded from mouse SAN cell. Local Ca^{2+} release events (red arrows) were frequent prior to each global Ca^{2+} transient. Lower panel shows the spatial averaging of Ca^{2+} dynamics from this image (arbitrary unit).

B, Surface-plot of the Ca^{2+} image from panel C. **Inset:** a transmission image of a typical isolated SAN cell. Red line shows the confocal laser scan line that runs longitudinally along the edge of the cell.

Figure 2. Coupling between SR Ca^{2+} release and membrane action potential in a SAN cell

A, Confocal line-scan Ca^{2+} imaging in a SAN cell loaded with Fluo-4 penta-potassium salt through a glass pipette. Arrows denote local Ca^{2+} sparks preceding action potential upstroke and Ca^{2+} transients. Note: Due to the limitation of confocal line imaging, localized release events were not always captured at the foot of each Ca^{2+} transient. **B**, Spatial average of Ca^{2+} signals in panel A. **C**, Corresponding membrane potential recording. The cell was current-clamped with no stimulus in perforated patch clamp configuration.

Figure 3. Mouse SAN cell displays stable graded CICR

A, Voltage protocol utilized for experiments. **B**, Confocal Ca^{2+} images from a mouse SAN cell, showing triggered Ca^{2+} sparks during the ramp depolarization (from -60 to -40 mV for 500 ms), global Ca^{2+} transients upon 300-ms step depolarization (test pulses, as shown, -30 mV, 0 mV, +10 mV, +30 mV, +50 mV), tail release, and spontaneous Ca^{2+} sparks at holding potential of -60 mV. **C**, Ca^{2+} profiles of confocal Ca^{2+} images from panel B. **D**, Corresponding $\text{I}_{\text{Ca,L}}$ current and NCX inward current (black arrows) related to each ramp depolarization (red frames included areas of panel B-C-D).

Figure 4. Gradation of $I_{Ca,L}$ and SR Ca^{2+} release in mouse SAN cells

A, $I_{Ca,L}$ density as a function of membrane potential ($n=8$); **B**, Amplitude of Ca^{2+} transients measured at different voltages (triggered by Ca^{2+} current of panel A) ($n=8$); **C**, Gain function as an index of the amplification of CICR in SAN cells ($n=8$). **D** Activation kinetics of Ca^{2+} transients in SAN cells and ventricular cardiomyocytes is similar. At 0 mV, $T_{peak}=35.0\pm5.08$ ms, $n=8$ for SAN cells; $T_{peak}=39.2\pm3.9$ ms, $n=5$ for ventricular myocytes, $p>0.05$.

Figure 5. Mouse SAN cells display frequent local Ca^{2+} release during ramp depolarization

A, Comparison of Ca^{2+} spark frequency during ramp and at holding potential of -60 mV. Pooled data are from 8 control SAN cells (10 sweeps/cell). The spark frequency during ramp stage is 5.7 times higher than recorded at holding potential, suggesting local Ca^{2+} release during phase 4 diastolic depolarization is largely (85%) contributed by triggered Ca^{2+} sparks. Mean $\pm$ SE, $n = 8$ (**, $p < 0.001$). **B**, Spatial average of the Ca^{2+} signals during ramp (10 images) from the same cell as shown partly in Fig 2A. The dashed arrows indicate corresponding voltages during the ramp pulse. In this cell, the sparks were initiated at ~ -54 mV ($F/F_0 = 1.1$ is defined as the level that local Ca^{2+} release is initiated). Similar results were obtained from 7 other SAN cells.

Figure 6. Characteristics of local Ca^{2+} release events (Ca^{2+} sparks) in SAN cells

Sparks were measured during ramp depolarization and at holding potential of -60 mV. **A**, Spark amplitude (F/F_0); **B**, Spark duration (FDHM, ms); **C**, Spark size (FWHM, μm); Error bars represented S.E.M., $n=445$ sparks for ramp group, $n=205$ sparks for -60 mV group. **D–F**, Distribution of SAN Ca^{2+} spark amplitude, duration, and width during ramp depolarization; **G–I**, Distribution of SAN Ca^{2+} spark amplitude, duration and width measured at -60 mV.

Figure 7. Slow Ca^{2+} chelator EGTA does not prevent the frequent occurrence of localized Ca^{2+} release during diastolic depolarization

A, Voltage clamp protocol; **B,** Membrane current (I_{Ca}) recording at test pulse 0 mV; **C,** A representative confocal line scan image along the periphery of a SAN cell, synchronized with I_{Ca} recording of panel B; **D,** Spatial average of SR Ca^{2+} release from panel C.