Rhythmic Ca^{2+} Oscillations Drive Sinoatrial Nodal Cell Pacemaker Function to Make the Heart Tick

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ABSTRACT: Excitation-induced Ca^{2+} cycling into and out of the cytosol via the sarcoplasmic reticulum (SR) Ca^{2+} pump, ryanodine receptor (RyR) and Na^{+}-Ca^{2+} exchanger (NCX) proteins, and modulation of this Ca^{2+}cycling by β-adrenergic receptor (β-AR) stimulation, governs the strength of ventricular myocyte contraction and the cardiac contractile reserve. Recent evidence indicates that heart rate modulation and chronotropic reserve via β-ARs also involve intracellular Ca^{2+} cycling by these very same molecules. Specifically, sinoatrial nodal pacemaker cells (SANC), even in the absence of surface membrane depolarization, generate localized rhythmic, submembrane Ca^{2+} oscillations via SR Ca^{2+} pumping-RyR Ca^{2+} release. During spontaneous SANC beating, these rhythmic, spontaneous Ca^{2+} oscillations are interrupted by the occurrence of an action potential (AP), which activates L-type Ca^{2+} channels to trigger SR Ca^{2+} release, unloading the SR Ca^{2+} content and inactivating RyRs. During the later part of the subsequent diastolic depolarization (DD), when Ca^{2+} pumped back into the SR sufficiently replenishes the SR Ca^{2+} content, and Ca^{2+}-dependent RyR inactivation wanes, the spontaneous release of Ca^{2+} via RyRs again begins to occur. The local increase in submembrane [Ca^{2+}] generates an inward current via NCX, enhancing the DD slope, modulating the occurrence of the next AP, and thus the beating rate. β-AR stimulation increases the submembrane Ca^{2+} oscillation amplitude and reduces the period (the time from the prior AP triggered SR Ca^{2+} release to the onset of the local Ca^{2+} release during the subsequent DD). This increased amplitude and phase shift causes the NCX current to occur at earlier times following a prior beat, promoting the earlier arrival of the next beat and thus an increase in the spontaneous firing rate. Ca^{2+} cycling via the SR Ca^{2+} pump, RyR and NCX, and its modulation by β-AR stimulation is, therefore, a general mechanism of cardiac chronotropy and inotropy.

KEYWORDS: beta-adrenergic receptor stimulation; local Ca^{2+} release; ryanodine receptor; sinoatrial nodal cells

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INTRODUCTION

Rhythmic intracellular Ca^{2+} cycling—not directly dependent on surface membrane potential—is an integral component of biological clocks that regulate diverse vital functions throughout nature.[1–5] A capacity to generate rhythmic changes in intracellular Ca^{2+} is imparted by organelles that can sequester and release Ca^{2+} at specific intervals. One type of intracellular Ca^{2+} cycling involves the endoplasmic reticulum or sarcoplasmic reticulum (SR), which is endowed with a Ca^{2+} pump and release channels that permit rhythmic cycling of Ca^{2+} into and out of this compartment. The periodicity of such Ca^{2+} cycling is regulated by the Ca^{2+} pump and Ca^{2+} release channel characteristics, their acute modulation by various signaling pathways, and the quantity of Ca^{2+} available for pumping. The gain of multiple cell signaling pathways can be modulated by changes in the periodicity of these Ca^{2+} rhythms (i.e., frequency modulation of an amplitude function).

The heart's pacemaker cells generate spontaneous rhythmic changes of their membrane potential, thereby producing roughly periodic electrical depolarizations or action potentials (APs). Pacemaker cells within the heart having "clocks" with the briefest periods "capture" or drive other excitable cells to depolarize the myocardium. Sinoatrial nodal pacemaker cells (SANCs) are the dominant cardiac pacemaker cells because they exhibit shorter periods between spontaneous APs than do atrioventricular nodal, or His-Purkinje cells. A role of specific sarcolemmal ion channels in the spontaneous membrane potential of cardiac pacemaker cells has been firmly established by decades of research applying electrophysiological techniques.[6–12] The role of intracellular Ca^{2+} releases in cardiac arrhythmias had previously been most appreciated,[13] but earlier studies,[14–16] however, had failed to embrace or had not emphasized a specific role for local increases in Ca^{2+} inside the cell membrane with respect to modulation of the pacemaker potential and the spontaneous beating rate of SANC. The advent of Ca^{2+} sensitive indicators, coupled to fluorescence microscopy or confocal imaging, and simultaneous measurement of membrane potential or current, has permitted investigation into the role of Ca^{2+} release in normal cardiac pacemaker function.[17–32]

INTRACELLULAR CALCIUM CYCLING

Localized, Submembrane Ryanodine Receptor Ca^{2+} Release during Diastolic Depolarization in SANC

We studied single, spindle-shaped, spontaneously beating SANC isolated from the rabbit hearts. The beating rate of these SANC (n = 118) was between 2 Hz and 5 Hz and their capacitance between 20 pF and 95 pF. Both beating rate and cell size were normally distributed around the average values of 3 Hz and 50 pF, respectively (FIG. 1A). There was no correlation between the spontaneous beating rate and cell size (FIG 1A).

Like other excitable cells, SANC cycle Ca^{2+} into and out of their SR.[19,24,25,28,32] Activation of ryanodine receptors (RyRs), the SR Ca^{2+} release channel, like that of many other classically described sarcolemmal ion channels, is cyclically regulated by time- and Ca^{2+}-dependent gating mechanisms.[33,34] Upon activation, RyRs release

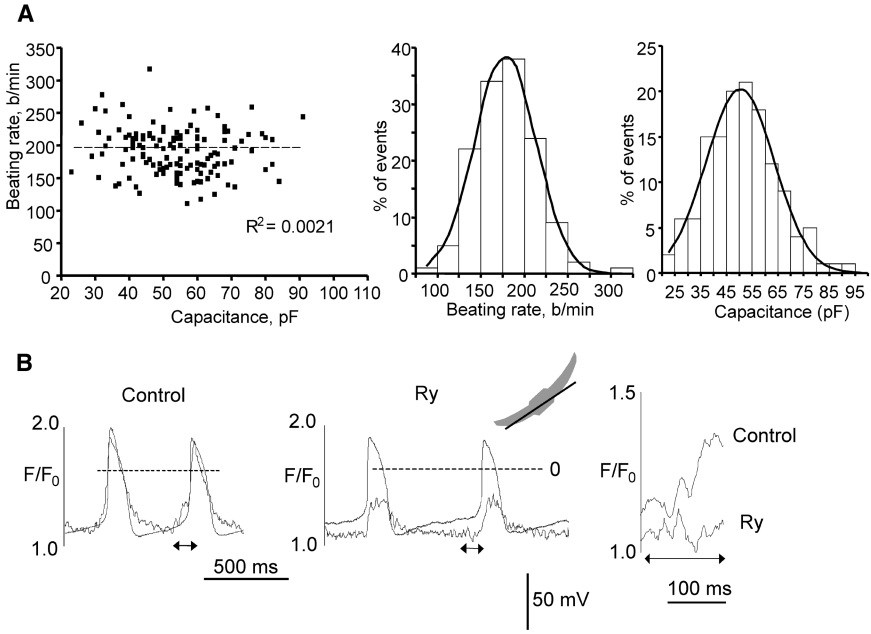

FIGURE 1. (**A**) Sinoatrial nodal pacemaker cells (SANC) size and beating rate: (*Top left*) Correlation between SANC (n = 118) size and beating rate; (*middle*) SANC beating rate histogram; (*right*) capacitance histogram. (**B**) Variation of subsarcolemmal Ca^{2+} release in the presence or absence of ryanodine (Ry). Simultaneous recordings of membrane potential and subsarcolemmal [Ca^{2+}] defined using confocal line-scan images of SANC (***inset***) in the presence or absence of 3 μmol/L Ry. F/F_0 is the instantaneous fluorescence of the Ca^{2+} indicator, fluo-3, relative to its minimal fluorescence (F_0), and other experimental conditions are as previously published.[19,25] Note that in the predrug control state, the Ca^{2+} waveform exhibits an increase during the later part of the spontaneous diastolic depolarization (DD) (*bracketed by arrows and shown at greater resolution in rightmost panels*). Thus, DD component of the Ca^{2+} waveform is abolished by Ry concurrent with the beating rate slowing.

Ca^{2+} into a narrow cleft beneath the SANC sarcolemma at two distinct times during each spontaneous SANC duty cycle (FIGS. 1B and 2A): in response to the AP rapid upstroke, as in ventricular myocytes, and during the later part of spontaneous diastolic depolarization (DD) before the next AP upstroke.

Local RyR Ca^{2+} Releases Do Not Require Membrane Depolarization

Studies in SANC that are voltage clamped at the maximum diastolic potential or permeabilized with saponin indicate that ignition of local Ca^{2+} releases (LCRs) during DD in spontaneously beating cells does not require the concomitant depolarization. LCRs occur in permeabilized cells and their characteristics vary with the bathing [Ca^{2+}] (FIG. 2B). Elevating free [Ca^{2+}] from 100 to 250 nmol/L results in

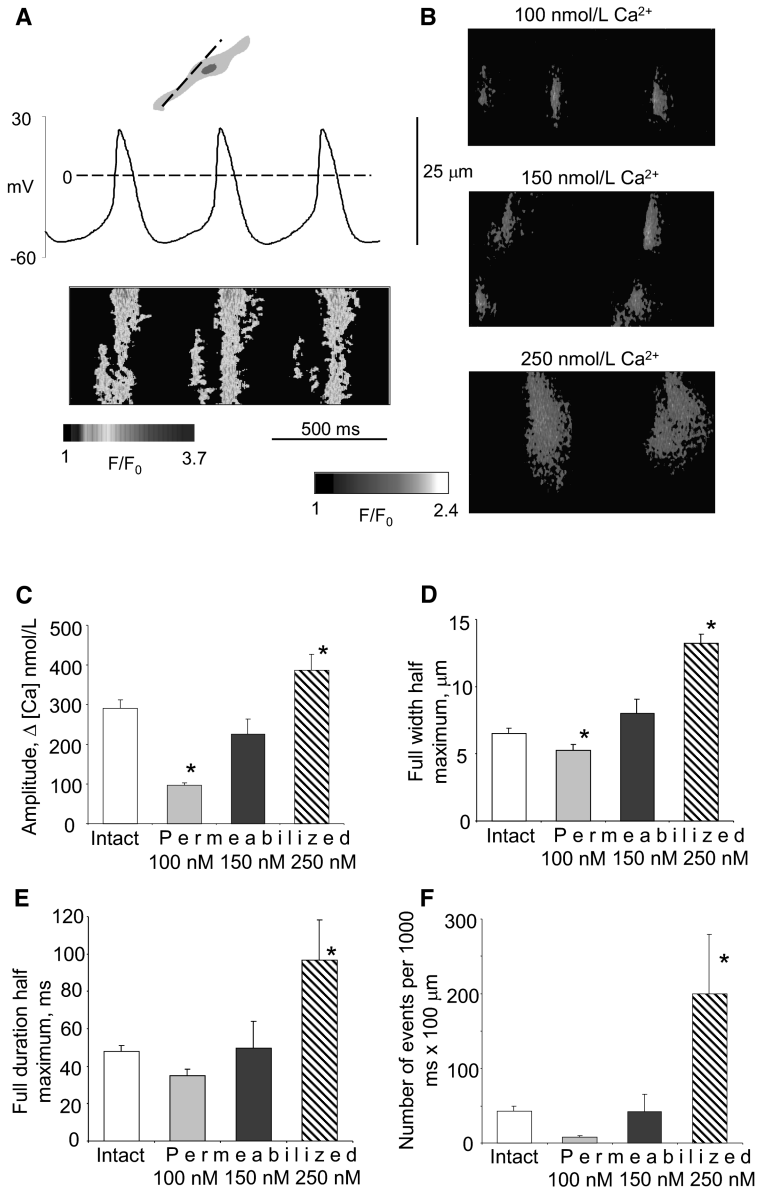

FIGURE 2. Local Ca^{2+} releases (LCRs) and comparison of their spatiotemporal properties in intact spontaneously beating and permeabilized sinoatrial nodal pacemaker cells (SANCs). (**A**) Recordings of action potentials (APs) and confocal line-scan images (see cell cartoon) in a representative, spontaneously beating SANC. (**B**) Confocal line-scan images in a "skinned" cell recorded with Fluo-4 K salt, in a representative "skinned" cell bathed in 100nmol/L, 150 nmol/L and 250 nmol/L [Ca^{2+}]. (**C–F**) Average characteristics of subsarcolemmal LCRs in intact, spontaneously beating cells and skinned cells.[32]

increases in the absolute LCR amplitude (FIG. 2C), spatial width (FIG. 2D), duration (FIG. 2E), and frequency (FIG. 2F). Calibration of fluo-3 fluorescence[32] estimated an average diastolic and systolic [Ca^{2+}] in intact cells of 160 ± 13 and 1150 ± 213 nmol/L, respectively. FIGURE 2C–2F also shows that spatiotemporal properties of LCRs in permeabilized cells at 150 nmol/L Ca^{2+} most closely resemble those in spontaneously beating cells.

Voltage clamping of SANC terminates spontaneous beating, which is restored on voltage clamp removal. FIGURE 3A illustrates a confocal line-scan image of subsarcolemmal Ca^{2+} in a representative, spontaneously beating SANC prior to and during acute voltage clamp at the maximum diastolic potential. The main point of the figure is that LCRs not only occur during DD during spontaneous beating, but also occur during voltage clamp, in the absence of a change in membrane potential. Similar LCRs were recorded when cells are paced under voltage clamp conditions with an AP waveform (to ensure SR Ca^{2+} loading) before voltage clamping of the membrane at –60 mV. The time during voltage clamp of each cell was divided into "would-be" cycles; that is, time intervals equal to the cycle length during spontaneous beating

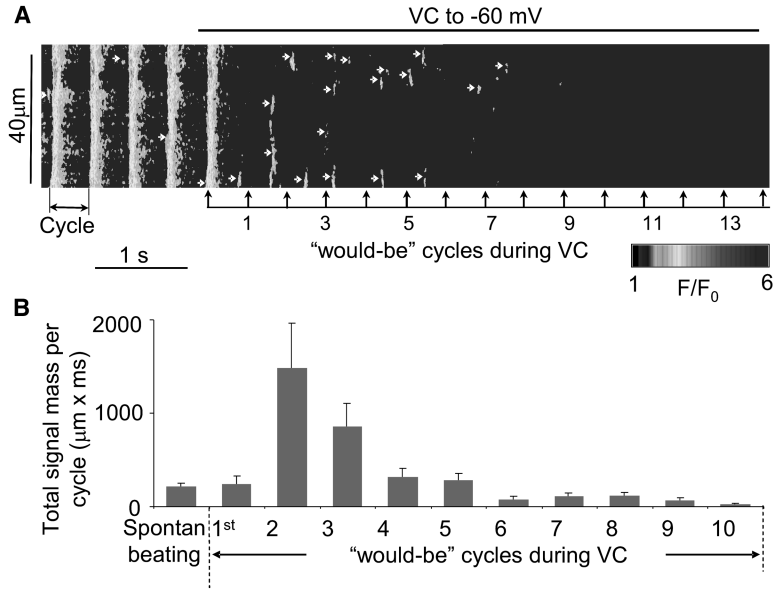

FIGURE 3. Local Ca^{2+} releases (LCRs) total signal mass within the cycle during spontaneous beating and during would-be inter-action-potential (AP) intervals during voltage clamp at the maximum diastolic potential. (**A**) Line-scan image of a representative sinoatrial nodal pacemaker cell (SANC) during spontaneous beating and during voltage clamp. *Horizontal arrowheads* indicate LCRs, and *vertical arrows* indicate would-be cycles during voltage clamp (i.e., times corresponding to the inter-AP interval if spontaneous firing were to have continued in the absence of voltage clamp). Local change in Ca^{2+} resulting from each LCR event during diastolic depolarization or during voltage clamp was characterized by its signal mass. (**B**) Average total LCR signal mass in 9 cells in control during spontaneous beating and during each would-be cycle of voltage clamp.[32]

(FIG. 3A). The first would-be cycle during voltage clamp is of particular significance, because at this time SR Ca^{2+} loading or RyR inactivation status would not be expected to differ from that during the prior spontaneous beating. FIGURE 3B demonstrates that the signal mass of local Ca^{2+} releases during the first would-be cycle does not differ from that during spontaneous beating.

Local RyR Ca^{2+} Releases Are Roughly Periodic

Since LCRs in intact SANC during voltage clamp or in SANC permeabilized with saponin do not require a depolarizing trigger, it is possible that they are a manifestation of an intracellular rhythmic or roughly periodic Ca^{2+} oscillator. FIGURE 4 shows that the fast Fourier transform (FFT) of the Ca^{2+} releases during voltage clamp (FIG. 4B) exhibits periodicity, with a dominant power at 2.9 Hz. Membrane current fluctuations accompanied Ca^{2+} fluctuations during the voltage clamp. The FFT of these current oscillations exhibits similar periodicity as that of LCRs, with the dominant power at 2.9 Hz (FIG. 4B). Importantly, the dominant period of 345 ms both in calcium and current oscillations during voltage clamp is about 20% shorter than the spontaneous cycle length of 435 ms in this cell before voltage clamp.

Periodicity of Local RyR Ca^{2+} Releases during Steady-State Spontaneous Beating is Linked to the Spontaneous Cycle Length

The spontaneous beating rate among isolated rabbit SANC varies over a substantial range of frequencies (FIG. 1A). If submembrane Ca^{2+} releases during spontaneous beating are rhythmic and their occurrence does indeed modulate the spontaneous beating frequency, then cells that beat faster ought to have shorter spontaneous LCR periods than those that beat at a slower rate. FIGURE 4D shows that the interval from the rapid upstroke of the Ca^{2+} transient caused by the prior AP to the upstroke of the subsequent LCR (FIG. 4C) directly correlates with the variation in the spontaneous cycle length among different cells. Importantly, regardless of the absolute spontaneous cycle length, which varies from cell to cell, LCRs predominantly occur at constant relative time (i.e., at 80% to 90% of that cycle length) (FIG. 4E). Most of the spontaneous local Ca^{2+} release occurs beneath the cell membrane during the later part of the DDs, producing an inward current to accelerate the terminal part of the DD and driving the membrane potential to reach threshold to fire on AP.

Na^+-Ca^{2+} Exchanger is a Crucial Element Linking LCRs to Membrane Depolarization

Although the experiments in FIGURES 2 and 3 demonstrate that LCRs in spontaneously beating cells do not require changes in membrane potential, there is still a possibility that they could be triggered by Ca^{2+} influx during spontaneous beating as well as during voltage clamp (e.g., by T-type Ca^{2+} current).[24] A comparison of LCRs during spontaneous beating before and after exposure to 50 μmol/L Ni^{2+} indicates no difference in the number of LCRs per cycle during spontaneous beating, and in all cells subjected to 50 μmol/L Ni^{2+} LCRs are not abolished during voltage clamp.[32]

Several time- and voltage-dependent ion currents become activated during the DD, and therefore may link RyR Ca^{2+} release from the SR to DD modulation. T-type and L-Type Ca^{2+} currents ($I_{Ca,T}$, $I_{Ca,L}$), hyperpolarization-activated inward current

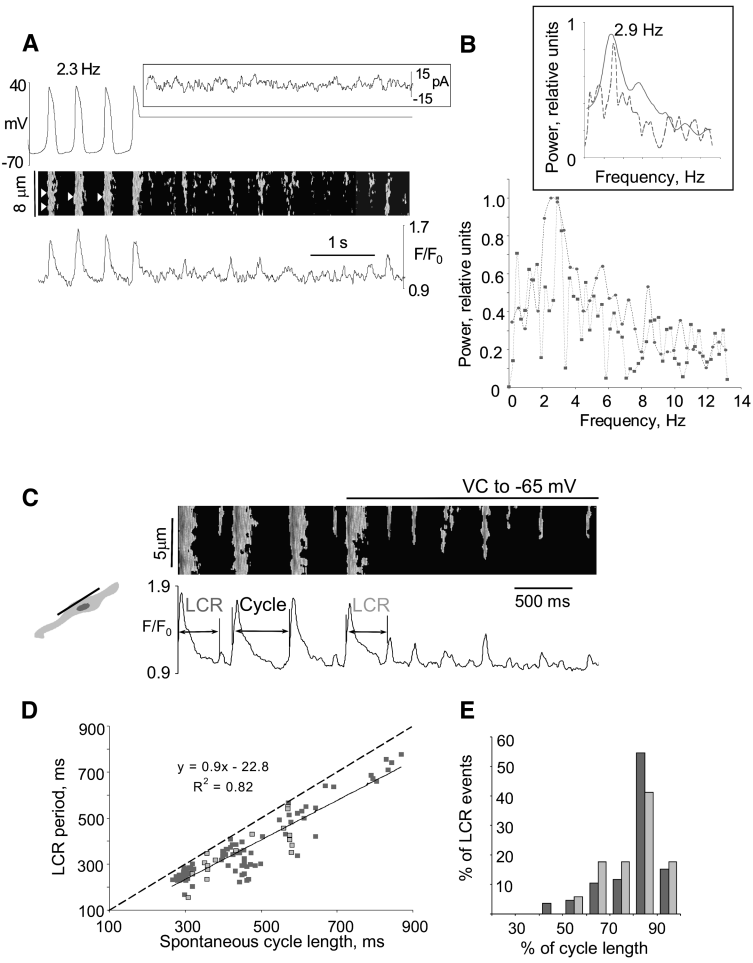

FIGURE 4. Local Ca^{2+} releases (LCRs) exhibit periodicity during voltage clamp and during spontaneous beating. (**A**) Simultaneous recordings of action potential (AP) (*top*), linescan image (*middle*), and normalized fluorescence (*bottom*) averaged over the image width in a representative cell before and during voltage clamp to -10 mV. ***Inset*** shows current oscillations recorded during voltage clamp. (**B**) Fast Fourier transform of Ca^{2+} waveform (*solid line*) and current oscillations (*dotted line*) shown in **A**. ***Inset*** depicts smoothed curves obtained with Gaussian filter. (**C**) Confocal linescan image (see cell illustration) and normalized subsarcolemmal fluorescence averaged over the image width in a representative sinoatrial nodal pacemaker cell measured before and during voltage clamp. *Double-headed arrows* delineate the LCR period during spontaneous beating and during the first "would-be" cycle during voltage clamp. (**D**) Periodicity of LCRs during spontaneous beating and during the first "would-be" cycle of voltage clamp: (black symbols) relationship between cycle length and the LCR period during spontaneous beating (10 cells, 86 LCRs); (gray symbols) the relationship between LCR period and the first "would-be" cycle during voltage clamp (13 cells, 17 LCRs). *Dashed line* is the line of identity. (**E**) (*black bars*) Probability of LCR occurrence as a function of the relative cycle length in cells in **D** during spontaneous beating; (*gray bars*) during the first "would-be" cycle during voltage clamp.[32]

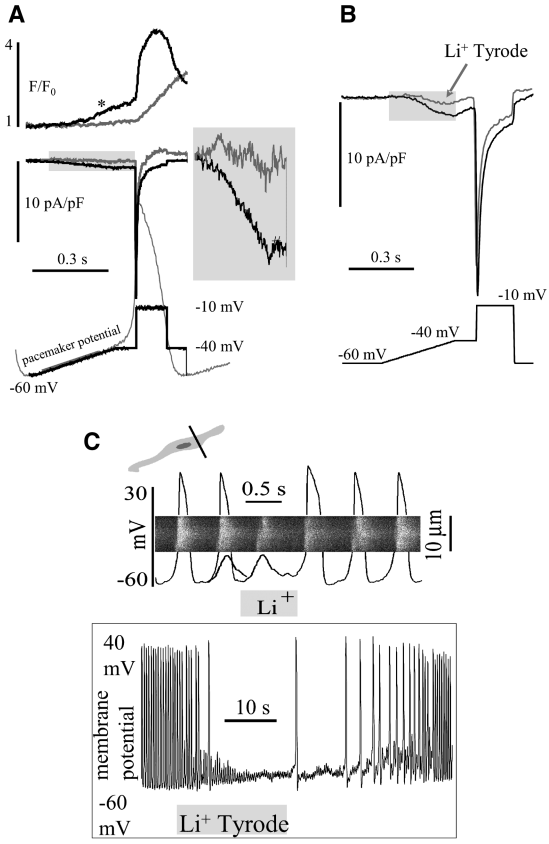

FIGURE 5. Effects of ryanodine (Ry) and Li^+ on inward current during diastolic depolarization (DD) and spontaneous beating in sinoatrial nodal pacemaker cells (SANCs). (**A**) Ry inhibits submembrane Ca^{2+} increase and inward current during the DD without affecting peak $I_{Ca,L}$. Normalized fluorescence (*top*) and membrane current (*middle*) under voltage clamp before (black) and 4 min after (gray) addition of 3 µmol/l ryanodine; voltage-clamp protocol is shown in *bottom panel*. *Middle right panel* displays the indicated part of the current record at greater magnification to more optimally show the inhibitory effect of Ry on inward current during the voltage ramp. (*) Local $[Ca^{2+}]_i$ increase in the absence of ryanodine and corresponding inward current. Both are inhibited in the presence of ryanodine. (**B**) Membrane current during voltage clamp before (black) and 10 s after (gray) superfusion with Na^+-free, Li^+-containing solution during voltage protocol (*bottom*). Highlighted area shows an inhibitory effect of Na^+-free Li^+ spritz on inward current during the voltage ramp. (**C**) (*Top*) Linescan image of Ca^{2+} release with superimposed action potential (AP) records during rapid and brief superfusion with a solution in which Na^+ was replaced by Li^+. Note that the maneuver blocked the subsequent AP firing. Scanned line was oriented perpendicular to long cell axis at half its depth (***inset***). Gray curve superimposed on the last AP preceding spritz of Na^+-free solution is a copy of the residual membrane potential oscillation observed during the Li^+ solution spritz. (*Bottom*) The inhibitory effect of Na^+ replacement by Li^+ on spontaneous beating in SANCs. AP recordings during onset and after washout of Na^+-free, Li^+-containing solution.[25]

(I_f), chloride current, the rapid component of delayed rectifier K^+ current (I_{Kr}), a time-independent background current carried by Na^+, and Na^+-Ca^{2+} exchanger (NCX) current all exhibit a Ca^{2+}-dependence. The effect of LCR occurrence during DD on the beating rate, however, is mediated by an inward current as ryanodine, which abolishes the diastolic Ca^{2+} release, suppresses the simultaneously measured inward current (FIG. 5A). Earlier studies had identified NCX current in cat latent pacemaker cells,[16] and this current has also been implicated in the spontaneous beating of toad pacemaker cells.[35] FIGURE 5B indicates that the electrogenic NCX (blocked by lithium) generates the Ca^{2+}-dependent inward current resulting from RyR Ca^{2+} release. Blockade of either the local Ca^{2+} release or the NCX should have potent effects on the spontaneous beating of these cells. An acute, rapid, transient application of lithium reduces the slope of the later DD and prevents the subsequent AP from firing without altering the diastolic RyR Ca^{2+} release (FIG. 5C, *top*). A longer exposure to Li^+ abolishes beating (FIG. 5C, *bottom*).

The magnitude of the ryanodine effect on adult pacemaker cell spontaneous beating rate has varied among studies (from 20% to 100%, review[28]). A critical dependence of cardiac pacemaker regulation on RyR Ca^{2+} release-NCX activation demonstrated in adult rabbit SANC becomes manifest early during development. The normal developmental increase in heart rate *in vitro* is markedly depressed in cardiac myocytes differentiated *in vitro* from embryonic stem (ES) cells with a functional knockout (KO) of the RyR2 (FIG. 6A), and these cells exhibit a markedly depressed late DD slope (FIG. 6C).[30] Exposure of wild-type ES-derived cardiomyocytes to ryanodine, which at low concentration locks RyRs in a subconductance state, imparts the characteristics of these cells to RyR KO cells (FIG. 6B). NCX KO mice die in embryo without evidence of the heart ever beating.[36]

Numerical Modeling of Submembrane Ca^{2+} Release Effect on SANC Beating Rate

Numerical modeling indicates that coupling of the RyR-generated Ca^{2+} waveform to the NCX current, when the latter is the only Ca^{2+}-dependent current operational during DD, is sufficient to increase the beating rate via DD acceleration.[28] A novel numerical model[31] that features local diastolic Ca^{2+} release component predicts that both the timing and amplitude of the RyR Ca^{2+} release during the DD determine its impact on SANC spontaneous beating rate (FIG. 7A–C). Thus, NCX is a partner of the RyR in amplification of DD and in modulation of the SANC beating rate.

We utilized a combination of experimental and numerical approaches to characterize how depolarizations produced by individual LCRs via NCX impact on the DD fine structure to control the spontaneous SANC firing rate.[37] Exponential LCR growth during the DD imparted a nonlinearity to its later part, which exhibited beat-to-beat potential (V_M) fluctuations, averaging about +2 mV. Numerical modeling, utilizing experimentally measured LCR amplitudes and delay times measured from the prior beat, simulated the stochastic ensemble of LCRs and respective NCX currents (FIG. 7D–F) that reproduced V_M fluctuations of the same magnitude and timing as those measured experimentally. The modeling also showed that the timing and amplitude of V_M fluctuations were determinants of the onset and amplitude of the nonlinear DD

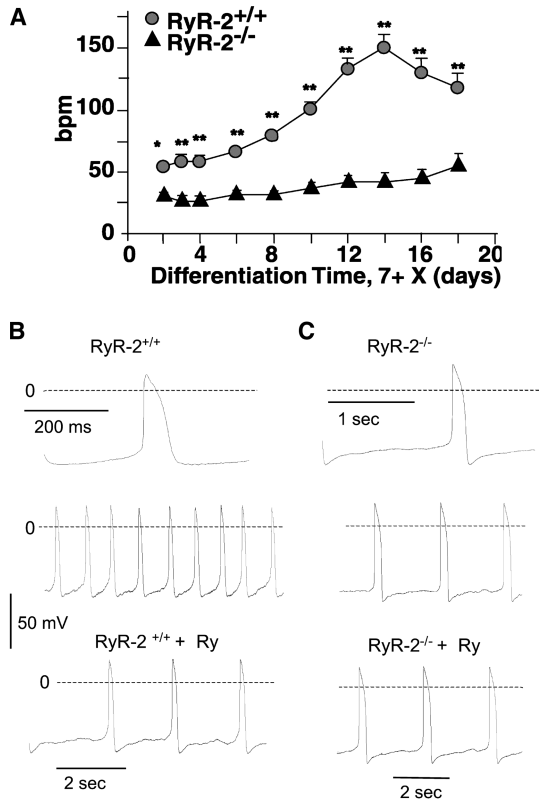

FIGURE 6. Spontaneous beating rates of ryanodine receptor (RyR)-$2^{+/+}$ and RyR-$2^{-/-}$ cardiomyocytes in embryoid bodies and single isolated cells in the absence and presence of ryanodine. (**A**) Beating rate in the RyR-$2^{-/-}$ cardiomyocytes within embryoid bodies and its increase with developmental time is significantly suppressed compared to RyR-$2^{+/+}$ cells. (**B, C**) Action potential recordings in representative RyR-$2^{+/+}$ and (**B**) RyR-$2^{-/-}$ cardiomyocytes. Firing rate of RyR-$2^{+/+}$ cell before (*middle*, 71 b/min) and after (*bottom*, 26 b/min) Ry (10 μmol/L). (**C**) The firing rate of RyR2 KO cardiomyocytes before (*middle*) and after (*bottom*) exposure to Ry did not change (26 b/min).[30]

component. Maneuvers that altered LCR timing or amplitude (i.e., ryanodine, BAPTA [1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)], nifedipine, or isoproterenol) similarly affected V_M fluctuations during the late DD, and the V_M fluctuation response evoked by these maneuvers was tightly correlated with the change in spontaneous beating rate. When changes in LCR properties and numbers are introduced into our new stochastic SANC model, the model predicts experimentally measured changes in the spontaneous beating rate induced by these maneuvers. Thus, ensemble of elementary NCX currents induced by LCRs links LCRs to the spontaneous V_M fluctuations and modulates the late, nonlinear DD fine structure, linking LCRs to the spontaneous SANC beating rate.[37]

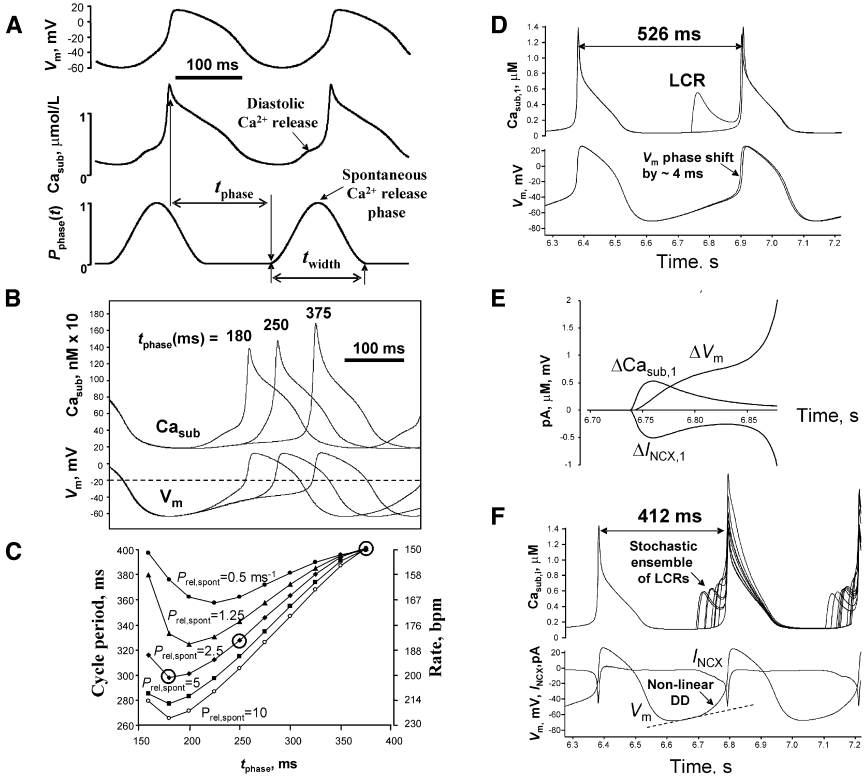

FIGURE 7. Diastolic calcium release controls the beating rate of rabbit sinoatrial nodal pacemaker cells (SANCs): numerical modeling of the coupling process. (**A**) Simulated spontaneous action potentials (APs), V_m and submembrane Ca^{2+}, Ca_{sub}. The spontaneous Ca^{2+} release flux was described as $j_{spont} = P_{phase}(t) \cdot P_{rel,spont} \cdot (Ca_{rel} - Ca_{sub})/[1 + (K_{rel}/Ca_{sub})^2]$, where $P_{phase}(t) = 0.5 - 0.5 \cdot \cos[2 \cdot \pi \cdot (t - t_{phase})/t_{width}]$ is the diastolic Ca^{2+} release phase function, t is time after the previous Ca^{2+} transient peak, $t_{width} = 150$ ms, $t_{phase} = 150$ ms, $P_{rel,spont} = 5$ ms^{-1} is the rate constant for the spontaneous Ca^{2+} release from the junctional sarcoplasmic reticulum. (**B**) Steady-state firing of APs, V_m, and respective Ca_{sub} transients simulated with varying phase of the diastolic release (t_{phase}). The simulated traces were shifted along the time axis to be in phase at maximum diastolic potential. (**C**) Both the phase and the amplitude of the diastolic Ca^{2+} release control the steady-state rate of AP firing. The family of plots shows relationships of cycle period (or rate) versus the phase of the diastolic release (t_{phase}) for various Ca^{2+} release amplitude determined by $P_{rel,spont}$ (in ms^{-1}). Circled data points correspond to simulations shown in panel **B**. (**D**) Introduction of only one local Ca^{2+} release (LCR) (*top*) into our recent stochastic sinoatrial nodal pacemaker cell model[31] results in AP upstroke phase shift towards earlier occurrence (*bottom*). (**E**) Unitary membrane depolarization (ΔV_m) and local unitary Na^+-Ca^{2+} exchanger (NCX) current ($\Delta I_{NCX,1}$) were determined as the difference (Δ) between simulations with and without an LCR occurrence shown in panel **D**. (**F**) Introduction of a stochastic ensemble of 40 LCRs results in significant shortening of the cycle length (426 ms vs. 526 ms, panel **D**) as the respective stochastic ensemble of inward-going unitary NCX currents (I_{NCX}) changes diastolic depolarization (DD) from linear to nonlinear fashion ("Nonlinear DD," *bottom*) reducing time to reach the threshold for L-type Ca^{2+} channel activation and thus the time at which the subsequent AP fires.[31,37]

Synchronization of Ryanodine Receptor Ca^{2+} Release in Sinoatrial Pacemaker Cells Underlies β-Adrenergic Receptor Modulation of Spontaneous Beating Rate

While it has long been recognized that β-adrenergic receptor (β-AR) stimulation increases the spontaneous beating rate of SANC, specific links between β-AR signaling and the resultant increase in firing rate have not been fully elucidated. Multiple ion channels are affected by β-ARs-cAMP signaling.[38–42] Given the more recently recognized role of diastolic RyR Ca^{2+} release in pacemaker cells spontaneous firing rates,[28,29] an important issue arises as to whether the increase in SANC beating rate effected by β-ARs also involves an effect on this Ca^{2+} release.

There is evidence to indicate that altered Ca^{2+} flux via RyR is indeed involved in the chronotropic effect of β-ARs in toad, guinea pig, and rabbit SANCs.[17–19,40] The total signal mass of Ca^{2+} released during DD reflects the total number of RyRs activated during a given DD. The signal mass of LCRs during diastole in SANC, and its phase, determine the impact of this Ca^{2+} release with respect to NCX activation on membrane potential depolarization and beating rate.[28,31] β-AR stimulation increases the number of local subsarcolemmal Ca^{2+} releases during a given DD (FIG. 8A and B) and also increases their amplitudes. Thus, synchronization of RyRs activation states (RyR recruitment) by β-AR stimulation in SANC leads to an increased number of RyRs participating in local diastolic Ca^{2+} release in SANC. This spatiotemporal synchronization of RyR Ca^{2+} release, due to synchronization RyR gating by β-AR stimulation, augments the inward current via NCX during the DD (FIG. 8C). Ryanodine prevents the effect of β-ARs to increase the diastolic Ca^{2+} release signal mass and the inward current it produces (FIG. 8C). The effect of β-AR stimulation to increase the spontaneous SANC firing rate is markedly blunted if normal RyR function is disabled (FIG. 8D), suggesting that modulation of Ca^{2+} release via RyR is an essential feature of optimal β-AR stimulation-induced acceleration of the SANC firing rate. In other terms, the RyR Ca^{2+} release during DD acts as a "switchboard" to link β-AR stimulation to an increase in SANC firing rate: recruitment of additional activated RyRs by β-AR stimulation, and partial synchronization of LCR occurrence leads to an increase in the heart rate. New evidence indicates that autonomic nervous signals to elicit tachycardia *in vivo* are decoded via ryanodine-sensitive, rhythmic Ca^{2+} cycling within pacemaker cells.[43]

Recent evidence indicates that constitutive protein kinase A (PKA) activation generates the rhythmic spontaneous Ca^{2+} oscillations in rabbit SANC and regulates intrinsic, spontaneous beating even in the absence of β-AR stimulation.[44] Constitutive β-AR activation is not a factor in the PKA activation dependence of beating rate, as neither the β_1-AR or β_2-AR blockers affect the beating rate. PKA inhibition substantially reduces phosphorylation of phospholamban (PLB) at serine 16, and confocal imaging of LCR shows that PKA inhibition also markedly increases the LCR period, and this effect is highly correlated with the concomitant reduction in the beating rate.[44] Stimulation of β-AR with isoproterenol produces changes opposite to PKA inhibition (i.e., PLB phosphorylation increases, LCR period decreases, and beating rate increases).

A modified version of our primary SANC model[31] simulated the effects of PKA inhibition.[45] When experimentally measured changes in both LCR (numbers and phase) due to PKA inhibition and sarcolemmal ion conductances (g_{CaL}, g_h, g_{Kr} decreased to 80% of control) were introduced into the model, the simulation faithfully

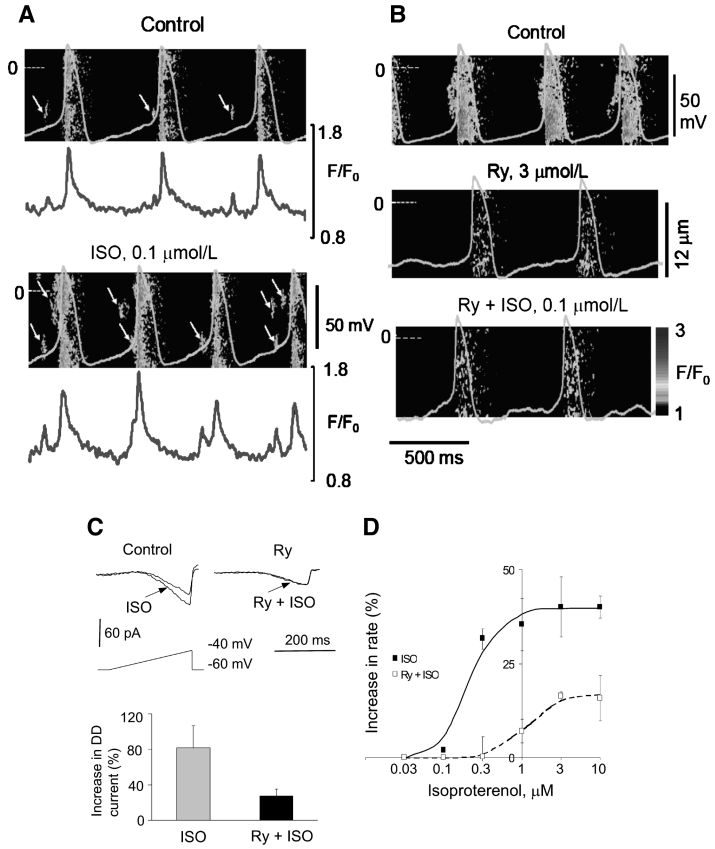

FIGURE 8. β-adrenergic stimulation effects on local ryanodine receptor (RyR) diastolic Ca^{2+} releases and diastolic depolarization (DD) current in sinoatrial nodal pacemaker cells (SANCs) with normal and disabled RyR function. (**A**) (*Top*) Simultaneous action potential (AP) recordings (gray curve) and linescan images of Ca^{2+} releases in the same cell, along the same scanline, before and during application of Isoproterenol. (*Bottom*) Normalized subsarcolemmal fluorescence. Isoproterenol (ISO) induced an increase in the number, brightness, and spatial width of local diastolic Ca^{2+} releases (*white arrows*), and this was accompanied by a 20% increase in the firing rate. (**B**) Simultaneous AP recordings and linescan images of Ca^{2+} releases along the same scanned line in control, after application of 3 μmol/L of ryanodine and of ryanodine + ISO. When RyRs are inhibited by ryanodine, local diastolic Ca^{2+} releases are suppressed, the firing SANC rate decreases, and β-adrenergic receptor (β-AR) stimulation fails to amplify local diastolic Ca^{2+} releases and fails to increase the SANC firing rate. (**C**) Effects of RyR inhibition on the ISO potentiation of inward current during a voltage clamp ramp simulating the DD in the SANC. *Top panels* show the voltage protocols and original current recordings for the simulated DD current. *Bottom panel* shows relative increase in mean DD current amplitude by 1 μmol/L ISO before (n = 5) and after block of RyR with 3 μmol/L. D, Effect of RyR inhibition on the concentration response of SANC firing rate to β-AR stimulation. ISO causes a dose-dependent increase in the firing rate with an apparent threshold concentration of 0.1 μmol/L. This effect is markedly decreased in the presence of ryanodine. The maximal effect of ISO occurred at 1 and 3 μmol/L in the absence and presence of ryanodine, respectively.[19]

reproduced the experimental effect on beating rate. Introduction into the model of changes induced in LCR by PKA inhibition alone produced a 23.2% effect, whereas introducing ion channel changes alone resulted in relatively small effect of 8.7%; normal rhythm was maintained in both cases. When sarcolemmal ion conductances were further decreased (i.e., to 66.7% of control without the LCR support), the beating rhythm became unstable and disrhythmic, similar to the experimental extremes during PKA inhibition. Introduction of LCRs into the model under the very same conditions restored the stable beating rhythm. Thus, the model predicts that LCRs occurring during the late phase of DD provide a powerful and timely prompt for the membrane to depolarize to the level of Ca^{2+}channel activation threshold, not only to regulate the beating rate but also to ensure a healthy rhythmic beat.[45]

Thus, constitutive PKA activation (i.e., not dependent on β-AR stimulation) generates intrinsic SANC rhythmicity; stimulation of β-AR extends the modulatory range of PKA activation.[44] The link between the spontaneous beating of SANC and synchronization of diastolic RyR Ca^{2+} release is in part attributable to cAMP-PKA-dependent phosphorylation of Ca^{2+} flux into the cell and Ca^{2+} pumping into the SR and release from SR, mediated by PKA-dependent phosphorylation of PLB, RyRs, and L-type Ca^{2+} channels. The potential role of a direct action of β-AR stimulation on NCX in pacemaker cells, to date, has not been determined.

Synchronization of RyR Ca^{2+} Release in Ventricular Cell Underlies β-ARs Modulation of Contractility

In ventricular myocytes, synchronization of L-type Ca^{2+} channel activation by cAMP-PKA signaling following depolarization, and synchronization of RyR Ca^{2+} releases generate a global cytosolic Ca_i transient of increased amplitude and accelerated kinetics. Thus, synchronization of RyR activation and synchronization of the ensuing Ca^{2+} release by cAMP-PKA signaling causes the heart to beat both stronger and faster. It follows, therefore, that such synchronization of RyR activation is a unified mechanism that links cardiac inotropy and chronotropy. In myocytes synchronization of RYR firing by cAMP-PKA signaling is manifest following AP (i.e., in response to activation of L-type Ca^{2+} current); whereas in SANC, it occurs prior to the AP and modulates activation of the NCX and the late DD rate, thus determining when the next AP will occur. An apparently similar or analogous mechanism that augments the beating rate of SANC leads to arrhythmias in ventricular cells.[46–49] Experimentally, however, β-AR stimulation-induced arrhythmias are usually observed when the same external pacing rate applied prior to β-AR stimulation also prevails during β-AR stimulation. In nature, however, β-AR stimulation modulates Ca^{2+} in both SANC and ventricular myocytes, and commensurate with the ensuing enhanced beating frequency (shorter diastole), the subsequent AP occurs at a sufficiently early time to "overdrive" the late diastolic release (i.e., to suppress its occurrence by activation of L-type channels which trigger RyRs to produce RyR Ca^{2+} release as a systolic event).[45,46]

Spontaneous SR Ca^{2+} Oscillations Generate Automaticity in Cardiac Cell Types other than Sinoatrial Nodal Pacemaker Cells

Spontaneous, RyR SR-generated oscillations of intracellular Ca^{2+} have previously been indirectly detected by laser light scattering technique; or recordings of

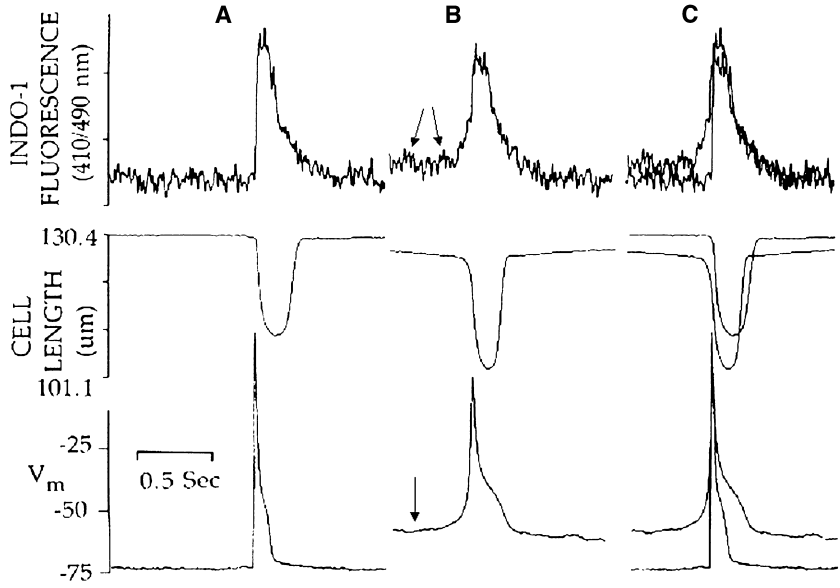

FIGURE 9. Spontaneous sarcoplasmic reticulum Ca^{2+} release triggers action potential (AP) in ventricular myocytes. (**A**) Electrical stimulator triggers an abrupt AP that generates a synchronized Ca^{2+}-triggered (INDO-1) fluorescence and contraction. (**B**) Spontaneous Ca^{2+} release (*arrow*) generates a slow change in membrane potential that reaches threshold to open Na^+ channels to trigger spontaneous AP. (**C**) Superimposition of A and B.[51]

voltage; or current, or myofilament motion or tension in unstimulated, multicellular cardiac muscle and Purkinje fibers (see Ref. 50 for review). With the advent of intracellular Ca^{2+} probes (see Refs. 50 and 51 for review) the underlying Ca^{2+} oscillations were subsequently directly demonstrated. These spontaneous Ca^{2+} oscillations originate locally within cells, and while stochastic they are roughly periodic and their periodicity (<0.1 Hz to ~7 Hz) varies with cell and SR Ca^{2+} loading. Further, during electrical stimulation, AP-triggered and spontaneous Ca^{2+} release are interactive with respect to their magnitude and occurrence (see Ref. 51 for review). In ventricular, atrial, and Purkinje fiber cells, spontaneous SR calcium oscillations produce delayed after-depolarizations[52] driven by a calcium-activated transient inward current that may initiate arrhythmias.[53] Spontaneous Ca^{2+} release of this sort leads to spontaneous depolarization of ventricular myocytes and is a cause of "abnormal automaticity" of these cells.[54] FIGURE 9 illustrates this phenomenon.

SUMMARY

On the basis of the evidence reviewed above, it can be envisioned that the spontaneous beating rate of pacemaker cells involves cyclic variation of subsarcolemmal Ca^{2+} produced by local releases of SR Ca^{2+} via RyRs, acting both in concert with

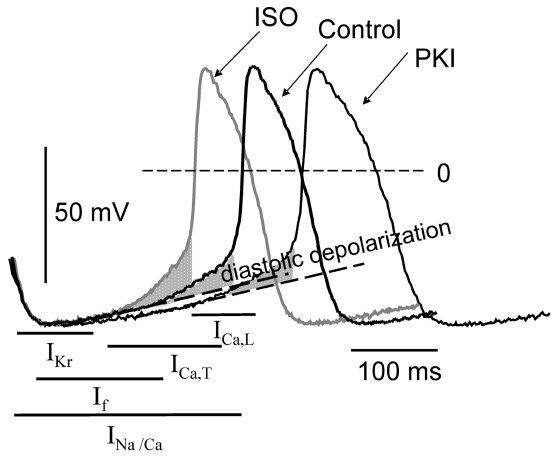

FIGURE 10. A schematic depicting the membrane potential and timing of the occurrence of the subsarcolemmal ryanodine receptor (RyR) Ca^{2+} release during the later part of diastolic depolarization (DD) (shaded area) of sinoatrial nodal pacemaker cells, before or after protein kinase A (PKA) activation by β-adrenergic receptor (β-AR) stimulation with isoproterenol (ISO) or PKA inhibition by a specific peptide inhibitor PKI (myristoylated protein kinase A inhibitor amide 14–22), and on the timing of activation of an ensemble of sarcolemmal ion channels as reported in the literature (in the absence of maneuvers that affect PKA activity). The *dashed line* is an extension of the rate of DD following the maximum diastolic potential. The deviation of the actual membrane potential from the *dashed line* occurs concomitantly with an increase in diastolic subsarcolemmal $[Ca^{2+}]$, due to RyR release (shaded areas). Following PKA activation by β-AR stimulation, this deviation exhibits a phase shift; that is, its initial trajectory occurs earlier than in control, due largely to enhanced Ca^{2+} activation of the Na^+-Ca^{2+} exchanger current, and it drives the membrane potential to the threshold required for action potential (AP) firing at an earlier time. Critical influences of I_f and I_{Kr} mostly occur prior to the period of the shaded area; $I_{Ca,T}$ is activated prior to and during the earlier part of the shaded area, and $I_{Ca,L}$ begins to activate during the later part of the shaded area and explosively activates during the rapid AP upstroke. Following suppression of PKA by PKI, Local Ca^{2+} release (LCR) amplitude is suppressed and LCR occurrence is delayed in time, leading to a decrease in the beating rate.

the NCX and with an ensemble of sarcolemmal ionic channels[6–12,29] to regulate the SANC spontaneous diastolic membrane depolarizations (FIG. 10). While no single factor likely confers the status of dominance with respect to pacemaker function of SANC, the rhythmic SR subsarcolemmal Ca^{2+} releases during the DD impart physiological robustness (stability) to the oscillating sarcolemmal ion currents[28] and strengthen responses to hormonal regulation.

The number of local Ca^{2+} releases within a given epoch (e.g., during DD in SANC, or following an AP in ventricular cells, or an increase in local Ca^{2+} release amplitude) is indicative of a recruitment of more RyRs to fire within that epoch. Such recruitment reflects a synchronization of individual RyR gating and Ca^{2+} release. Synchronization of RyR activation and Ca^{2+} release is a common mechanism that underlies the cAMP-PKA signaling to increase both heart rate and myocardial contraction amplitude. In SANC, the integral of the total number of elementary RyR

Ca^{2+} releases during a given epoch (i.e., during DD) and the elementary inward currents via NCX that these generate, determine the magnitude (amplitude) of the effect of these releases on the change in membrane potential during DD.

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