Response to Research Commentary

Cyclic Variation of Intracellular Calcium
A Critical Factor for Cardiac Pacemaker Cell Dominance

Edward G. Lakatta, Victor A. Maltsev, Konstantin Y. Bogdanov, Michael D. Stern, Tatiana M. Vinogradova

Abstract—While a diversity of cell types and distribution within the sinoatrial node and cell-cell interactions add complexity to a complete elucidation of the heart’s pacemaker function, it has become clear that cyclic variation of submembrane [Ca\(^{2+}\)] and activation of the Na\(^+-\)Ca\(^{2+}\) exchanger during diastolic depolarization (DD) act in concert with ion channels to confer on sinoatrial node cells (SANCs) their status of dominance with respect to pacemaker function. Studies using confocal microscopy indicate that subsarcolemmal Ca\(^{2+}\) release via ryanodine receptors occurs not only in response to the action potential (AP) upstroke, but also during the DD, and this is augmented by β-adrenergic receptor (β-AR) stimulation. Spontaneous APs simulated by mathematical SANC models beat at a faster rate when this subsarcolemmal Ca\(^{2+}\) waveform measured under β-AR stimulation is introduced into the modeling scheme. Thus, in future investigation of pacemaker functioning in health, disease, and disease therapies the “bar ought to be raised” to embrace the impact of cyclic variation in submembrane [Ca\(^{2+}\)] on pacemaker function. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2003;92:e45-e50.)

Key Words: sinoatrial node ■ β-adrenergic stimulation ■ ryanodine receptor ■ submembrane Ca\(^{2+}\) release

In pacemaker parlance, “dominance” refers to those excitable cells with “clocks” having the briefest periods, thereby allowing these cells to “capture” or drive other excitable cells, leading to a heart beat. Sinoatrial node cells (SANCs) achieve a dominant status because they exhibit shorter intervals between spontaneous action potentials (APs) than do atrial, atrioventricular nodal, or His-Purkinje cells. In SANCs, as in most excitable cells, voltage- and time-dependent transmembrane ion fluxes are also regulated by ion channels and carriers and their modulation by intracellular Ca\(^{2+}\) and phosphorylation. Studies using intracellular Ca\(^{2+}\)-sensitive indicators, coupled to confocal imaging and simultaneous measurement of membrane potential or current, have been interpreted to indicate that cyclic variation in submembrane Ca\(^{2+}\) coupled to Na\(^+-\)Ca\(^{2+}\) exchanger (NCX) activation modulates this SANC beating rate and is a factor in establishing SANC dominance in pacemaker function. Like other excitable cells, SANCs cycle Ca\(^{2+}\) into and out of their sarcoplasmic reticulum (SR). 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. Upon activation, RyRs release Ca\(^{2+}\) into a narrow cleft beneath the SANC sarcolemma at two distinct times during each spontaneous SANC duty cycle (Figure 1): 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. Several time- and voltage-dependent ion currents that become activated during the DD, ie, T-type and L-type Ca\(^{2+}\) currents (\(I_{\text{Ca},T}, I_{\text{Ca},L}\), hyperpolarization-activated inward current (\(I_{\text{h}}\)), chloride current, the rapid component of delayed rectifier K\(^{+}\) current (\(I_{\text{K}}\)), a time-independent background current carried by Na\(^+\), and NCX are Ca\(^{2+}\)-dependent and therefore may link RyR Ca\(^{2+}\) release from the SR to DD modulation. In their Research Commentary “Sarcoplasmic Reticulum Ca\(^{2+}\) Release Is Not a Dominating Factor in Sinoatrial Node Pacemaker Activity,” Honjo et al question the relevance of RyR Ca\(^{2+}\) release to cardiac chronotropy and its modulation by β-adrenergic receptor (β-AR) stimulation.

What Role Does RyR Ca\(^{2+}\) Release-NCX Activation Have in Pacemaker-Cell Dominance?
A critical dependence of cardiac pacemaker regulation on RyR Ca\(^{2+}\) release-NCX activation becomes manifest early during development. The normal increase in heart rate with differentiation is markedly depressed in cardiac myocytes differentiated in vitro from embryonic stem (ES) cells with a functional knockout (KO) of the RyR2 that exhibit markedly depressed DD slope. Exposure of wild-type ES cells to ryanodine (Ry), which at low concentration locks RyRs in a subconductance state, confers on these cells the properties of RyR KO cells. NCX KO mice die in embryo without evidence of the heart ever beating.
release with Ry reduces the spontaneous beating rate. Disabling the NCX experimentally prevents spontaneous SANC firing.

The effect of Ry to concurrently abolish the component of subsarcolemmal Ca\(^{2+}\) release during the DD and reduce the spontaneous beating rate in a representative rabbit SANC is shown in Figure 1. All studies that have used Ry in cardiac pacemaker cells or tissue, have, in fact, observed a reduction of the spontaneous beating rate of pacemaker cells or tissue (Table 1). In our opinion, the most important finding of Honjo et al\(^4\) is that Ry, indeed, reduced the spontaneous firing rate in isolated intact sinoatrial node (SAN) and single SANC, by 19% and 22%, respectively (Table 1), indicating that RyR Ca\(^{2+}\) release is indeed a critical determinant of the maximum beating rate and therefore, of their dominant pacemaker status.

**β-AR Stimulation Chronotropy in Pacemaker Cells**

β-AR stimulation, a potent physiological mechanism to accelerate the beating rate of pacemaker cells, recruits additional RyRs to release Ca\(^{2+}\) during the DD,\(^19\) activating NCX current to a greater extent, and thus increasing the DD amplitude (Figure 1, bottom traces). Several recent studies\(^15,20–22\) indicate that the full effect of β-AR stimulation to enhance the rate of pacemaker depolarization and to increase the beating rate, does, in fact, require intact RyR function (Table 2). Specifically, partial inhibition of normal RyR function by low concentrations of Ry, which results in SR Ca\(^{2+}\) depletion, blunts the effect of isoproterenol (ISO) to increase the beating rate, but does not always abolish the ISO chronotrophic effect.\(^15,19–22\) The link between the β-AR chronotropic effect and cyclic variation in cell Ca\(^{2+}\) is attributable, in part, to a β-AR stimulation–induced increase in protein kinase A–dependent phosphorylation of L-type Ca\(^{2+}\) channels, phospholamban, and RyRs. An increase in Ca\(^{2+}\) flux of L-type Ca\(^{2+}\) channels during β-AR stimulation also accelerates removal of L-type Ca\(^{2+}\) channel inactivation by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and enables these channels to be more responsive to a given DD.\(^18\) Thus, at least two Ca\(^{2+}\)-dependent mechanisms, removal of L-type channel inactivation via CaMKII and RyR Ca\(^{2+}\) release with Ry reduces the spontaneous beating rate. Disabling the NCX experimentally prevents spontaneous SANC firing.\(^9\)

### Table 1. Effect of Ry on the Spontaneous Beating Rate of Isolated SAN Tissue or Single SANC

<table>
<thead>
<tr>
<th>Species, Preparation (Reference No.)</th>
<th>Ry Concentration, μmol/L</th>
<th>Pre-Ry Beating Rate, bpm (mean±SEM)</th>
<th>Ry-Induced Decrease in Beating Rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit, SAN tissue strips (12)</td>
<td>1</td>
<td>213±20</td>
<td>20</td>
</tr>
<tr>
<td>Guinea pig, SAN (15)</td>
<td>2</td>
<td>221±13</td>
<td>39</td>
</tr>
<tr>
<td>Rabbit, SAN (4)</td>
<td>30</td>
<td>125±8</td>
<td>19</td>
</tr>
<tr>
<td>Cane toad, SVC (21)</td>
<td>2–10</td>
<td>31±2</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit, SANC (13)</td>
<td>1</td>
<td>181±9</td>
<td>25</td>
</tr>
<tr>
<td>Rabbit, SANC (9)</td>
<td>1</td>
<td>205±8</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit, SANC (9)</td>
<td>3</td>
<td>200±9</td>
<td>52</td>
</tr>
<tr>
<td>Rabbit, SANC (9)</td>
<td>30</td>
<td>202±9</td>
<td>95</td>
</tr>
<tr>
<td>Guinea pig, SANC (15)</td>
<td>2</td>
<td>145±52</td>
<td>88</td>
</tr>
<tr>
<td>Rabbit, SANC in cultured cells (11)</td>
<td>10</td>
<td>93±5</td>
<td>33</td>
</tr>
<tr>
<td>Rabbit, SANC (4)</td>
<td>30</td>
<td>216±12</td>
<td>22</td>
</tr>
</tbody>
</table>

SAN indicates intact sinoatrial node; SANC or SVC, freshly isolated SA nodal or sinus venous cells.
release to augment an inward current via NCX activation to augment depolarization, complement each other with respect to modulation of the DD and the beating rate of SANCs in response to β-AR stimulation. Ca\(^{2+}\)-dependent modulation of other SANC ion channels likely occurs during β-AR stimulation, but the relevance of this with respect to the β-AR stimulation–induced increase in spontaneous beating rate requires further study.

Honjo et al.\(^4\) in contrast to the results of multiple studies (Table 2), maintain that the effect of β-AR stimulation to increase the pacemaker firing rate does not require intact RyR function. They report that ISO could still augment the spontaneous beating rate in the presence of Ry. Generally, this is not inconsistent with the studies in Table 2, but because Honjo et al. studied only a single ISO dose after exposure to Ry, and because no data are provided on the effect of ISO before Ry, their results cannot be directly compared with these other studies. It is noteworthy that the very high concentration of Ry (30 \(\mu\)mol/L) used by Honjo et al. is higher than that in most previous studies (Table 2). Interestingly, one previous study found that ISO could overcome the effects of high (but not low) concentrations of Ry.\(^{21}\)

### Can a Single Factor Within Pacemaker Cells Confer Dominance?

A reductionist approach attempts to dissect the interactions that occur among a multitude of factors involved in pacemaking to determine which factor that influences membrane potential is the most important with respect to pacemaker function. In this context, Honjo et al.\(^4\) declare that SR Ca\(^{2+}\) release is not a dominating factor in SAN pacemaker activity because RyRs reduce the beating rate of isolated SA nodal tissue or cells they have isolated from the SA node by 20%. It is clear from the results of studies in Tables 1 and 2 that although classically studied ion currents are required to ensure spontaneous firing of SANCs, and critically contribute to the dominance of their pacemaking activity, none of these channels, per se, is sufficient to ensure a maximal firing rate (dominance) under a given ambient condition. Cyclic Ca\(^{2+}\) release via RyRs, however, is required to achieve the most rapid beating rate that can occur. Whether interference with RyR Ca\(^{2+}\) release reduces beating rate of the SA node by 20% or 100% (Table 1) is irrelevant. Thus, in contrast to Honjo et al.,\(^4\) we interpret their data and that prevalent in the literature (Tables 1 and 2) to conclude that the RyR Ca\(^{2+}\) release-NCX activation is necessary, but not sufficient, for pacemaker cells to achieve dominance.

### Diversity of Ca\(^{2+}\) Dependence of Cell Types Within the SAN

There is some evidence to indicate that cells within the SAN are not unique in nature or distribution\(^{24,25}\) and that the dominance of a given cell type within the SAN may shift to other cell types in response to various stimuli, eg, β-AR stimulation. Honjo et al.\(^5\) suggest that SANC type diversity may provide a plausible explanation for the differences observed among studies in Table 1 with respect to the effectiveness of Ry to influence the spontaneous beating rate and its response to β-AR stimulation. Specifically, they suggest that cells in the central area of the SAN, ie, sometimes referred to as “primary pacemaker cells,” have fewer RyRs than do other cells within the SAN and are therefore less Ca\(^{2+}\)-dependent than other SANC types. That Ry induced only a 20% decrease in the beating rate of the cells on which they report\(^3\) has been interpreted to indicate that these cells likely represent central or primary pacemaker cells and exhibit little Ca\(^{2+}\) dependence of their beating rate. Although this hypothesis is plausible, it would have been instructive if Honjo et al. were also to have examined whether chelation of intracellular Ca\(^{2+}\), eg, with intracellular BAPTA,\(^{18}\) or whether abrogating the effectiveness of the “partner” of the RyR, ie, the NCX, reduced the beating rate of cells they had studied. In short, it is our opinion that additional studies are required to precisely delineate the primary pacemaker site within the SAN in the study of Honjo et al.\(^5\) and to define the extent to which Ca\(^{2+}\)-dependent mechanisms affect the beating rate of different cell types comprising the SAN.

### Are Contemporary Pacemaker Models Lacking a Subsarcolemmal Ca\(^{2+}\) Waveform Sufficiently Robust to Define Pacemaker Cell Dominance?

Another basis for the position of Honjo et al.\(^5\) that SR Ca\(^{2+}\) release is unimportant in primary pacemaker cells arises from observations that mathematical model simulations of these cells exhibit spontaneous beating in the absence of consideration of a cyclicly varying subsarcolemmal Ca\(^{2+}\) waveform. Their numerical pacemaker model\(^{26}\) and most other models do not embrace the well-known concept of Ca\(^{2+}\)-dependent modulation of ion channels and in extreme cases do not consider cyclic changes in intracellular [Ca\(^{2+}\)] (or other ion

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### Table 2. Effect of Ry on the Increase in Pacemaker Beating Rate After β-AR Stimulation

<table>
<thead>
<tr>
<th>Species, Preparation (Reference No.)</th>
<th>Concentration of β-AR Agonist, (\mu)mol/L</th>
<th>Ry Concentration, (\mu)mol/L</th>
<th>Relative Decrease in the Efficiency of β-AR Agonist to Increase Beating Rate After Ry, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cane toad, SVC (21)</td>
<td>ISO, 2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit, SANC (22)</td>
<td>ISO, 1</td>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td>Rabbit, SANC (19)</td>
<td>ISO, 1</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>Human atrial cells (20)</td>
<td>Epinephrine, 10</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Guinea pig, SANC (15)</td>
<td>ISO, 0.1</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Guinea pig, SAN (15)</td>
<td>ISO, 0.1</td>
<td>2</td>
<td>34</td>
</tr>
</tbody>
</table>

SAN, SANC, and SVC are defined as in Table 1.
concentrations), assuming the intracellular \([\text{Ca}^{2+}]\) to be constant. To test whether a submembrane \([\text{Ca}^{2+}]\) waveform is a crucial determinant of the dominant pacemaker status of central SANCs studied by Honjo et al, we have integrated a \([\text{Ca}^{2+}]\) waveform measured in our experiments by confocal microscopy in an ISO-stimulated SANC, ie, similar to that in Figure 1 to their own numerical model for primary pacemaker cells. We observed that the spontaneous APs generated by the model were quickly entrained and driven to beat at a 17% higher rate, ie, at the rate of oscillatory \([\text{Ca}^{2+}]\) waveform that we integrated into their model (Figure 2). This result directly refutes the conclusion of Honjo et al that intact RyR function is not required in central SANCs for a fuel response to \(\beta\)-AR stimulation. We also found similar accelerating effects of an oscillating \([\text{Ca}^{2+}]\) waveform on AP firing rate in a numerical SANC model by Demir et al.

Moreover, we observed that the numerical model for primary pacemaker cells presented by Zhang et al, which does not embrace an oscillatory \([\text{Ca}^{2+}]\) waveform, lacks physiological robustness, because it fails to generate rhythmic APs when the initial membrane voltage is set within a physiologically relevant range, ie, close to \(-50\) mV, with background \([\text{Ca}^{2+}]\) held constant at different levels (Figure 3). However, when an experimentally measured \([\text{Ca}^{2+}]\) waveform similar to the control SANC \([\text{Ca}^{2+}]\) waveform in Figure 1 is integrated into the model, robust beating is restored in the context of the very same initial conditions. Figure 3 also illustrates the fact that \([\text{Ca}^{2+}]\) oscillations, per se, and not a steady \([\text{Ca}^{2+}]\), are important for the \([\text{Ca}^{2+}]\)-dependent effect to permit the generation of repetitive APs under these initial conditions, because the study in the aborted beating in Figure 3A was set at the same level (265 nmol/L) as the average \([\text{Ca}^{2+}]\) in Figure 3B. This suggests to us that a cyclic variation in \([\text{Ca}^{2+}]\) is required for robust maintenance of spontaneous beating, under some conditions, at least.

The marked effects of introduction of the \([\text{Ca}^{2+}]\) waveform into the model simulations in Figures 2 and 3 could be explained by a low density of many aforementioned sarcolemmal ion channels in central SANCs, which have a very small \(-20\)-pF electrical capacitance. In such cells, even a relatively small change in ion current balance, eg, due to an
The two spontaneous beats before initiation of external stimulation reveal the occurrence of the subsarcolemmal \( \text{Ca}^{2+} \) increase during the DD (A), which drives the trajectory of membrane potential versus calcium concentration \((F/F_0)\) in a counterclockwise loop (B). Note that by beat 5, the pre-AP increase in \( \text{Ca}^{2+} \) is precluded by the early occurrence of the externally driven AP, shifting the trajectory of membrane potential versus \( \text{Ca}^{2+} \) to a clockwise loop, similar to that seen during the duty cycle of normal ventricular myocytes (C).

**Figure 4.** External pacemaker captures the beating rate of an isolated, spontaneously beating SANC. A, Line-scan image of fluo-3 fluorescence with superimposed APs of the first and last beats in a representative SANC. External electrical stimulation through a patch pipette was initiated at beat 3, at a rate just higher than the spontaneous beating rate (see black arrows). The two spontaneous beats before initiation of external stimulation reveal the occurrence of the subsarcolemmal \( \text{Ca}^{2+} \) increase before or after the subsarcolemmal \( \text{Ca}^{2+} \) increase during the DD, as reported in the literature. The dashed line is an extension of the DD after the maximum diastolic potential. The deviation of the actual membrane potential from the dashed line occurs concomitantly with an increase in subsarcolemmal \([\text{Ca}^{2+}]\), due to RyR release (shaded areas). After ISO, this deviation exhibits a phase shift, i.e., its initial trajectory occurs earlier than in control, due largely to enhanced \( \text{Ca}^{2+} \) activation of the NCX current, and drives the membrane potential to the threshold required for AP, firing at an earlier time. Critical influences of \( i_l \) and \( i_h \) mostly occur before the period of the shaded area; \( i_{\text{Ca,L}} \) is activated before and during the earlier part of the shaded area, \( i_{\text{Ca,L}} \) begins to activate during the later part of the shaded area and explosively activates during the rapid AP upstroke.

Increased \( \text{Ca}^{2+} \)-dependent current via NCX activation (Figure 2C) can notably change the total membrane electrical charge, resulting in a significant change of membrane potential. Thus, the ion current balance in these cells may be such that even fewer RyR or NCX molecules are required in central versus peripheral SANCs with respect to \( \text{Ca}^{2+} \) modulation of their firing rate. Although the results of Figures 2 and 3 show that an intracellular \( \text{Ca}^{2+} \) waveform is a critical factor in determining modeled pacemaker dominance, the contrary (that beating rate and dominance are adequately explained without considering changes in intracellular \( \text{Ca}^{2+} \)) cannot be argued because of the relatively primitive state of pacemaker modeling. Existing models are based on phenomenological (rather than microscopically validated) representations of individual currents, neglecting important phenomena such as ion-dependent inactivation and mode shifts of \( \text{Ca}^{2+} \) channels. Because spontaneous APs are the result of a delicate balance of currents, large changes in the beating rate (or cessation of beating altogether) can be produced by modest changes in model parameters. Models have often felt free to adjust parameters to achieve the desired rates. Presently, there are no SANC models that incorporate a modern understanding of the mechanisms of intracellular \( \text{Ca}^{2+} \) via RyRs or the role of local intracellular \( \text{Ca}^{2+} \) gradients. It is our opinion that much work remains to be done to bring state-of-the-art pacemaker models to the stage where they can be used to test physiological questions.

**Overdriving the Cyclic \( \text{Ca}^{2+} \) Waveform of SANCs**

From Figures 1, 2, and 3, any external depolarizing influence (e.g., that emanating from an artificial pacemaker or that arising from tweaking of pacemaker model parameters) that occurs after a prior AP with a period shorter than that of the DD component of the \( \text{Ca}^{2+} \) waveform might be expected to override the effect of the subsarcolemmal \( \text{Ca}^{2+} \) increase during DD, “capture” the beating frequency, and thus become a critical factor with respect to pacemaker dominance. Figure 4 shows that the beating rate of a SANC can indeed be captured by an external pacemaker that “overdrives” \( \text{Ca}^{2+} \) releases during DD, changing the instantaneous relationship between the changes in subsarcolemmal \([\text{Ca}^{2+}]\) and membrane potential.

**Figure 5.** Schematic to depict the late diastolic component of the subsarcolemmal \( \text{Ca}^{2+} \) and membrane potential of SANCs, before or after \( \beta-\text{AR} \) stimulation by ISO, on the basis of previously published results,9–10 and the timing of activation of an ensemble of sarcolemmal ion channels (in the absence of ISO), as reported in the literature. The dashed line is an extension of the DD after the maximum diastolic potential. The deviation of the actual membrane potential from the dashed line occurs concomitantly with an increase in subsarcolemmal \([\text{Ca}^{2+}]\), due to RyR release (shaded areas). After ISO, this deviation exhibits a phase shift, i.e., its initial trajectory occurs earlier than in control, due largely to enhanced \( \text{Ca}^{2+} \) activation of the NCX current, and drives the membrane potential to the threshold required for AP, firing at an earlier time. Critical influences of \( i_l \) and \( i_h \) mostly occur before the period of the shaded area; \( i_{\text{Ca,L}} \) is activated before and during the earlier part of the shaded area, \( i_{\text{Ca,L}} \) begins to activate during the later part of the shaded area and explosively activates during the rapid AP upstroke.

**Summary**

On the basis of the results of many recent studies8–15,19–23 in addition to our recent studies9,10 featured in the Research Commentary of Honjo et al, we envision that the maximum spontaneous beating rate of pacemaker cells (dominance) is a result of cyclic variation of subsarcolemmal \( \text{Ca}^{2+} \), produced by the release of \( \text{Ca}^{2+} \) from the SR via RyRs acting in concert with the NCX and an ensemble of sarcolemmal ionic channels to regulate the SANC DD oscillations (Figure 5). The cyclic changes in \( \text{Ca}^{2+} \) during the DD impart physiological robustness (stability) to the oscillating sarcolemmal ion currents and strengthens responses to hormonal regulation. Because RyR \( \text{Ca}^{2+} \) release occurs simultaneously with spontaneous DD before the rapid upstroke of the subsequent AP in SANCs, it has been suggested that voltage-dependent \( \text{Ca}^{2+} \)
currents in at least one pacemaker cell type act as a trigger for this Ca\textsuperscript{2+} release.\textsuperscript{9} Voltage-independent Ca\textsuperscript{2+} release via RyRs, however, has also been demonstrated to occur within other cardiac cell types (see review \textsuperscript{28}). In ventricular myocytes, for example, voltage-independent SR Ca\textsuperscript{2+} release can induce sarcoplasmic DD mainly via Ca\textsuperscript{2+} modulation of NCX exchanger, resulting in “abnormal automaticity.”\textsuperscript{29} A similar voltage-independent mechanism of RyR Ca\textsuperscript{2+} release, therefore, may also exist within SANCs to modulate their “normal automaticity.” Additional studies to probe this hypothesis are warranted.

References

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