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New evidence for coupled clock regulation of the normal automaticity of sinoatrial nodal pacemaker cells: bradycardic effects of ivabradine are linked to suppression of intracellular Ca^{2+} cycling:

Dual effects of ivabradine on Ca^{2+} and membrane clocks

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Abstract

Beneficial clinical bradycardic effects of ivabradine (IVA) have been interpreted solely on the basis of I_f inhibition, because IVA specifically inhibits I_f in sinoatrial nodal pacemaker cells (SANC). However, it has been recently hypothesized that SANC normal automaticity is regulated by crosstalk between an “M clock,” the ensemble of surface membrane ion channels, and a “ Ca^{2+} clock,” the sarcoplasmic reticulum (SR). We tested the hypothesis that crosstalk between the two clocks regulates SANC automaticity, and that indirect suppression of the Ca^{2+} clock further contributes to IVA-induced bradycardia.

IVA ($3\mu\text{M}$) not only reduced I_f amplitude by $45\pm 6\%$ in isolated rabbit SANC, but the IVA-induced slowing of the action potential (AP) firing rate was accompanied by reduced SR Ca^{2+} load, slowed intracellular Ca^{2+} cycling kinetics, and prolonged the period of spontaneous local Ca^{2+} releases (LCRs) occurring during diastolic depolarization. Direct and specific inhibition of SERCA2 by cyclopiazonic acid (CPA) had effects similar to IVA on LCR period and AP cycle length. Specifically, the LCR period and AP cycle length shift toward longer times almost equally by either direct perturbations of the M clock (IVA) or the Ca^{2+} clock (CPA), indicating that the LCR period reports the crosstalk between the clocks. Our numerical model simulations predict that entrainment between the two clocks that involves a reduction in I_{NCX} during diastolic depolarization is required to explain the experimentally AP firing rate reduction by IVA.

In summary, our study provides new evidence that a coupled-clock system regulates normal cardiac pacemaker cell automaticity. Thus, IVA-induced bradycardia includes a suppression of both clocks within this system.

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Disclosures

None.

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Keywords

Sinoatrial nodal pacemaker cells; Ca^{2+} cycling; ion channels; physiology; sarcoplasmic reticulum

1. Introduction

Results of recent clinical trials (BEAUTIFUL, SHIFT, VIVIFY, etc.) indicate that a reduction in heart rate by ivabradine (IVA) was anti-anginal and anti-ischemic (for review cf. [1]). Moreover, IVA has been demonstrated to reduce diastolic dysfunction and cardiac fibrosis [2] and to increase exercise capacity [3]. In a model of myocardial ischemia/reperfusion, IVA improved the regional myocardial blood flow and function and reduced the infarct size [4]. In isolated sinoatrial nodal pacemaker cells (SANC) under voltage clamp and buffered Ca^{2+} conditions (i.e., presence of EGTA in the patch pipette), IVA, in low doses, specifically inhibits I_f of SANC but not other membrane currents [5, 6]. The beneficial clinical bradycardic effects of IVA have been interpreted solely on the basis of I_f inhibition [1–4, 7].

However, accruing evidence has been interpreted to indicate that normal automaticity of the SANC is regulated by integrated functions within a system of two clock-like oscillators [8–10]: the sarcoplasmic reticulum (SR), acting as a “ Ca^{2+} clock,” rhythmically discharges diastolic local Ca^{2+} releases (LCRs) beneath the cell surface membrane; LCRs activate an inward current (likely that of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger) that prompts a surface membrane or “M clock,” the ensemble of sarcolemmal electrogenic molecules, to effect an action potential (AP) (Fig. 1). Periodicity of Ca^{2+} clock-generated LCRs is regulated by the SR Ca^{2+} pumping, which depends not only on SR proteins (phospholamban (PLB) and ryanodine receptors), and their phosphorylation status, but also on function and phosphorylation status of M clock proteins, e.g., L-type Ca^{2+} channels that regulate cell Ca^{2+} available for SR pumping. According to the coupled-clock hypothesis [11], the M and Ca^{2+} clocks should crosstalk. Possibilities for crosstalk and feedback via changes in SR Ca^{2+} cycling include Ca^{2+} -dependent electrogenic processes (such as $\text{Na}^+/\text{Ca}^{2+}$ exchange) and voltage-dependent Ca^{2+} fluxes (such as via L-type Ca^{2+} channels). Thus, a change in AP firing rate in response to any disturbance signal that perturbs either clock entrains the function of the other clock. This is followed by a feedback from the entrained clock to the originally perturbed clock. Such feedback amplifies the response of the initially perturbed clock to the original signal, ensuring a robust response to the initial disturbance signal. Therefore, the steady-state AP cycle length change embodies contributions of both clocks, as in other coupled oscillatory systems throughout nature [12]. Because both M and Ca^{2+} clock molecules regulate cellular Ca^{2+} homeostasis, and resultant LCR characteristics, the coupled-clock hypothesis also predicts that in response to any chronotropic perturbation, the net changes in the steady-state LCR period are a reflection of integrated clock function.

Although the coupled-clock hypothesis is conceptually attractive, direct experimental evidence to support crosstalk between the two clocks is lacking, and the idea that normal automaticity of SANC is regulated by a coupled-clock system remains controversial. We sought to provide evidence for crosstalk between the two clocks by determining whether the effect of IVA, at a concentration that specifically inhibits I_f of the M clock to reduce the AP firing rate, is accompanied by effects on the intracellular Ca^{2+} cycling, i.e. on the “ Ca^{2+} clock.”

We further approached this clock crosstalk by determining the ability for observed changes in Ca^{2+} cycling to feed back to the M clock, by specific perturbation of the Ca^{2+} clock (using pharmacological inhibition of SR Ca^{2+} pump). We also further explore the detailed

mechanisms of the crosstalk using numerical model simulations that embrace AP, I_f , $I_{Ca,L}$ and Ca^{2+} -dependent phosphorylation effect on SR Ca^{2+} loading and release. Our discovery of the Ca^{2+} component in the bradycardic effects of IVA in cardiac pacemaker cells is clinically relevant, because this indicates that targeting the coupled-clock system within pacemaker cells, rather than a single effector molecule, such as funny channel, is likely to be a more general and effective strategy to normalize heart rate in patients requiring chronotropic therapy. Moreover, our results reveal the presence of powerful feed-forward and feed-back coupling mechanisms in SANC, providing new evidence that coupled-clock functions confer robustness to normal pacemaker cell automaticity.

2. Methods

To test the hypothesis that the AP cycle length is regulated by crosstalk between surface membrane ion channels and intracellular Ca^{2+} cycling, we superfused single, isolated rabbit SANC with IVA, a direct and specific blocker of I_f or Cyclopiazonic acid (CPA) a direct and specific inhibitor of Ca^{2+} pumping by SERCA2. We measured AP cycle length, cytosolic Ca^{2+} , SR Ca^{2+} load and LCR characteristics during diastolic depolarization in spontaneously beating SANC, and also LCR characteristics in permeabilized SANC. In addition, we measured I_f and $I_{Ca,L}$ in voltage-clamped SANC. We determined whether simulations of our mathematical model could reproduce the experimental results, i.e., whether the change in AP cycle length in response to IVA requires crosstalk between membrane ionic currents and SR Ca^{2+} fluxes. A detailed expanded description of experimental and numerical methods is available in the Online Data Supplement. The experiment protocols have been approved by the Animal Care and Use Committee of the National Institutes of Health (protocol #034LCS2013).

3. Results

3.1 IVA blocks I_f in SANC

We first verified whether the previously determined effects of 3 μ M IVA to inhibit I_f via whole cell voltage clamp in rabbit SANC [6] are reproducible in single, isolated rabbit SANC studied under our conditions. A representative example of the effect of IVA (3 μ M) on I_f over a range of membrane potentials is shown in Fig. 2A, and the average I_f characteristics in the presence or absence of IVA are summarized in Table S3. The average effects of IVA (n=9) on the I-V relationships of peak I_f are shown in Fig. 2B. At the maximum diastolic depolarization (MDP) during spontaneous AP firing ($\sim$ -59mV; Table S4), IVA decreased peak I_f on average, by $45\pm 6\%$ control (Fig. 2B inset). I_f activation (Fig. 2C) was calculated from the peak tail current 5 min after IVA application (i.e., during steady state I_f blockade). On average, IVA decreased peak I_f without significantly shifting its voltage-dependent activation (Fig. 2D, Table S3). Note that in spontaneously beating SANC, unlike in the case of voltage clamp, a long time between spontaneous APs might increase I_f activation that may contradict the effect of IVA to reduce I_f amplitude at MDP.

3.2 Effects of IVA on the SANC spontaneous AP firing rate, intracellular Ca^{2+} cycling and SR Ca^{2+} load

To determine the effect of IVA on AP firing rate and AP parameters, we superfused single SANC with IVA for 10 min. Representative examples of APs and average AP firing rate are illustrated in Figures 3A–B, respectively. AP parameters in the presence or absence of IVA are summarized in Table S4. On average (n=9) IVA reduced the AP-firing rate by $14\pm 2\%$ (from 153 ± 10 to 130 ± 10 beats/min). Note, that in time-control experiments in the absence of drug (n=9), the time-dependent decrease in AP firing rate was only $2.68\pm 3\%$ (not shown).

We next determined whether the effect of IVA to reduce the SANC AP firing rate is accompanied by an effect on intracellular Ca^{2+} cycling in single SANC loaded with Fluo-4 AM. Figure 3C illustrates the AP-induced Ca^{2+} transient and spontaneous diastolic LCRs (arrows) in a representative SANC. Table S5 lists the average characteristics of the AP-induced Ca^{2+} transients. The reduction in spontaneous AP firing rate in response to IVA is accompanied by a prolongation (by $12 \pm 5\%$) of the 90% decay time of intracellular Ca^{2+} (T_{-90_c}), while peak systolic Ca^{2+} (amplitude), time to peak Ca^{2+} and 50% decay time of intracellular Ca^{2+} (T_{-50_c}) were not significantly altered.

The spatiotemporal characteristics of diastolic LCRs determine the timing and extent of diastolic activation of an inward I_{NCX} , which accelerates the rate of diastolic depolarization. The average effects of IVA on these characteristics are listed in Table S5. IVA shifted the LCR size distribution to a smaller value (Fig. 3D), reduced LCR duration, reduced the average number of LCRs (normalized to 100 μm cell length and 1-s time interval), and markedly reduced ensemble LCR Ca^{2+} signal. The LCR period, defined as the time from the peak Ca^{2+} transient (the onset SR Ca^{2+} release triggered by the prior AP) to the LCR onset (as illustrated in Fig. 3C), was prolonged by IVA (Fig. 3E). Thus, changes in the LCR period in response to IVA occur concomitantly with changes in T_{-90_c} , and T_{-90_c} and the LCR period are highly correlated with each other both prior to and during exposure to IVA (Fig. 3E). These results indicate that, in conjunction with its bradycardic effect, IVA has a substantial effect to suppress spontaneous Ca^{2+} signaling.

To estimate whether LCR suppression is associated with a reduction in the SR Ca^{2+} content, brief, rapid pulses of caffeine were applied (“spritzed”) onto control cells and cells in the presence of IVA. Representative examples and average effects of caffeine induced Ca^{2+} release are presented in Figure 4A and Figure 4B, respectively. IVA significantly reduced the peak amplitude of the caffeine-induced Ca^{2+} transient by 13% (Fig. 4B).

3.3 Direct inhibition of SR Ca^{2+} pumping by CPA suppresses intracellular Ca^{2+} cycling and prolongs AP cycle length

To determine whether the IVA effect on Ca^{2+} cycling and SR Ca^{2+} load could be reproduced by direct perturbation of the Ca^{2+} clock, we employed 0.5 μM CPA which directly and specifically inhibits SERCA2 Ca^{2+} pumping in order to reduce the SR Ca^{2+} load [13] to a similar extent as that effected by IVA. A representative example of the CPA on the caffeine-induced Ca^{2+} transient is shown in Figure 4C. Fig. 4D shows that, on average, CPA significantly reduced the SR Ca^{2+} load, indexed by the amplitude of the caffeine-induced Ca^{2+} transient, by 20%.

Fig. 5A shows the effects of CPA on the AP-induced Ca^{2+} transient and on LCRs in a representative SANC. Table S5 lists the average characteristics of the AP-induced Ca^{2+} transients and LCR characteristics measured by Fluo-4. As in the response to IVA, the peak systolic Ca^{2+} (amplitude), time to peak Ca^{2+} and T_{-50_c} were not significantly altered, but also as in response to IVA, T_{-90_c} was prolonged (by $12 \pm 4\%$) in response to CPA. The effects of CPA on LCR characteristics were also similar to those in response to IVA: LCR size distribution shifted to smaller values (Fig 5B) and the ensemble LCR Ca^{2+} signal was reduced by CPA and on average, the LCR period was prolonged by $13 \pm 2\%$ by CPA. Thus, as was the case for IVA, changes in LCR period in response to CPA occurred concomitantly with changes in the T_{-90_c} , and the two changes in parameters are highly correlated (Fig. 5C, Fig. S1). Fig. 5D illustrates the effect of CPA on AP cycle length in a representative SANC. Fig. 5E shows that, on average, a direct effect on intracellular Ca^{2+} cycling by CPA was accompanied by a reduction of the spontaneous AP firing rate (by $15 \pm 2\%$; $n=9$, from 155 ± 10 to 130 ± 10 beats/min), i.e., similar in magnitude of AP firing rate reduction effect of IVA. Table S4 shows that CPA effects on AP characteristics are also similar to those of

IVA. A comparison of Figs. 3, 4 and 5 and the data in Tables S4-S6 indicates that the effects of IVA and CPA on intracellular Ca^{2+} cycling and AP firing rate are strikingly similar.

3.4 Both IVA and CPA effects on LCR period and AP cycle are correlated

It has been previously documented that changes in AP cycle length in response to numerous perturbations that affect both the M and Ca^{2+} clocks (a decrease in cAMP/PKA levels, β adrenergic receptor stimulation, etc.) are linked to the LCR period [14, 15]. Fig. 6 demonstrates that spontaneous AP cycle length prior to and in response to either IVA (panel A) or CPA (panel B) is predicted by the concurrent LCR period. When data prior to and in response to these perturbations (IVA and CPA) in panels A and B are merged, they conform to a linear function (panel C) with the same slope as the control-IVA (panel A) and control-CPA (panel B). Moreover changes in LCR period among cells (from 335 ± 7 ms to 367 ± 7 ms for IVA from 342 ± 5 to 376 ± 8 ms for CPA), and concurrent changes in spontaneous SANC AP cycle length induced by IVA or CPA, form a linear function that lies near a line of identity (Fig. 6D, $r^2=0.9$, $p<0.001$). Thus, the LCR period is an integrated function of the coupled-clock system (because its relationship to the AP cycle length in response to IVA is identical to that in response to CPA).

3.5 IVA directly and specifically affects only the membrane clock and CPA directly and specifically affects only the Ca^{2+} clock

To prove that IVA does not directly suppress cell Ca^{2+} cycling, we measured the effect of IVA on $I_{\text{Ca,L}}$, an important determinant of coupled-clock function of cell Ca^{2+} loading in intact SANC. We also measured Ca^{2+} kinetics in permeabilized SANC. Representative examples of the effect of IVA on $I_{\text{Ca,L}}$, measured at -5 mV, and on average I-V relationship of $I_{\text{Ca,L}}$ are presented in Figures S2A and Figure S2B, respectively. Average $I_{\text{Ca,L}}$ characteristics are listed in Table S6. Peak $I_{\text{Ca,L}}$ in the presence of IVA did not differ from control (-13.7 ± 1 vs -13.3 ± 1 pA/pF, $n=12$). Furthermore, the reduction in $I_{\text{Ca,L}}$ in the presence of IVA did not statistically differ from $I_{\text{Ca,L}}$ time-control for run-down ($4.3 \pm 1\%$, $n=9$). Note that measurements of $I_{\text{Ca,L}}$ under voltage clamp and buffered Ca^{2+} may not mimic exactly how this ion current changes when intracellular Ca^{2+} changes during spontaneous AP firing.

We examined the effects of IVA on SR Ca^{2+} cycling by measuring LCR characteristics in permeabilized SANC. IVA did not affect LCR characteristics (Fig. 7, Table S7), and therefore did not have a direct effect to suppress SR- Ca^{2+} cycling. Specifically, IVA did not significantly change LCR frequency (number of LCR for $100 \mu\text{m}$ in 1 sec), duration, amplitude or size. Moreover, neither Ca^{2+} signal of individual LCR nor the LCR ensemble Ca^{2+} signal significantly changed in response to IVA. Therefore, $3 \mu\text{M}$ IVA employed to specifically and directly inhibit I_f did not directly suppress intracellular Ca^{2+} cycling.

To prove that CPA suppression of intracellular Ca^{2+} is not due to a direct effect on the M clock ionic currents we measured I_f and $I_{\text{Ca,L}}$, an important determinant cell Ca^{2+} loading. Representative examples of the CPA effect on $I_{\text{Ca,L}}$, measured at -5 mV, and the average effect on the I-V relationship of $I_{\text{Ca,L}}$ are presented in Figures S2C and Figure S2D, respectively. Average $I_{\text{Ca,L}}$ characteristics are listed in Table S6. Peak $I_{\text{Ca,L}}$ in the presence of CPA in voltage-clamped cells did not differ from control (-14.1 ± 2 vs -13.7 ± 2 pA/pF, $n=12$). Moreover, in voltage-clamped SANC, CPA did not affect the I_f peak or its kinetics (Table S3, Figure S3). Therefore, $0.5 \mu\text{M}$ CPA employed to specifically and directly inhibit SERCA pump did not directly affect the M clock.

3.6 A numerical model predicts cross talk in response to IVA

We employed a SANC numerical model developed previously [16, 17] in order to dissect contributions to the steady-state reduction in AP firing rate in response to IVA that result from (1) change in I_f , (2) feed-forward effect of IVA-induced cycle length increase to reduce Ca^{2+} influx (per unit time) and, hence, on SR Ca^{2+} pumping and release, and feedback of these Ca^{2+} cycling changes on AP firing rate, (3) additional feedback from change in intracellular Ca^{2+} cycling to Ca^{2+} -calmodulin activation of Adenylyl-cyclases (AC)/cAMP-PKA and Ca^{2+} /calmodulin protein kinase II (CaMKII) signaling. Please refer to the online supplement for details of the model and model parameters.

Fig. 8 illustrates the model simulations of the effects of 3 μM IVA on I_f AP, intracellular Ca^{2+} , $I_{\text{Ca,L}}$, junctional and network SR Ca^{2+} , and sarolemmal Na^+ - Ca^{2+} exchange current. The blue traces show the coupled-clock model simulations in the absence of IVA. The red traces show the model simulations when IVA directly affects I_f parameters (I_f parameters were changed according to our experimental results, see Table S1d for changes in model parameters). The model shows that a reduction in AP firing rate (Fig. 8B) in response to a decrease in I_f amplitude affects Ca^{2+} influx, SR Ca^{2+} cycling and the activation of I_{NCX} . A reduction in Ca^{2+} influx per unit time accompanies the reduction in AP firing rate via less frequent $I_{\text{Ca,L}}$ activation (due to a fewer number of simulated beats per unit time). The $I_{\text{Ca,L}}$ integral per 10 sec decreases from 194 in the absence of IVA to 183 $\text{pA}\cdot\text{s}$. Panels D to F in Fig. 8 illustrate the changes in cytosolic Ca^{2+} , junctional and network SR Ca^{2+} , respectively. Model simulations predict only a modest decrease in the peak cytosolic Ca^{2+} transient (by 2%), maximal Ca_{jsr} (by 0.2%) and maximal Ca_{nsr} (by 3%). This reduction in SR load is less than that measured experimentally in the steady state (Fig. 4). Moreover, the model predicts that IVA prolonged the T-90_c (by 8%). On the basis of its effects on cytosolic Ca^{2+} characteristics, IVA shifts the timing of diastolic depolarization at which inward sarcolemmal Na^+ - Ca^{2+} exchanger current was Ca^{2+} activated to a later time (by 7.5%), which matches the decrease in AP firing rate predicted by this model simulation. The effect of IVA on the I_{NCX} is not direct, but occurs via the resultant change in intracellular Ca^{2+} in response to IVA. Using a coupled-clock model [16, 17], we explored the relationship between the I_{NCX} , I_f and the membrane potential during diastolic depolarization. The blue tracings in Fig. 9 show the coupled-clock model simulations prior to IVA application. The red tracings in Fig. 9 show the model simulations in which IVA is present, but IVA only induces direct effects to reduce I_f i.e., in the absence of changes in the Ca^{2+} calmodulin AC-cAMP/PKA and CaMKII signaling to PLB. Note, that when IVA only directly affected I_f parameters, the I_{NCX} integral during diastolic depolarization (-65 to -45 mV) indeed became significantly reduced: from 221 $\text{pA}\cdot\text{mV}$ in control to 204 $\text{pA}\cdot\text{mV}$ and the I_f integral during diastolic depolarization reduced from 30 to 19 $\text{pA}\cdot\text{mV}$. Note, that these red simulations exclude any changes in the Ca^{2+} -calmodulin activation of AC-cAMP/PKA or CaMKII signaling that leads to changes in PLB-phosphorylation (change in K_{up}). In other words, the red model simulation considers only the change in Ca^{2+} balance due to the inseparable reduction in Ca^{2+} influx in the $I_{\text{Ca,L}}$ integral as reflected in less SR Ca^{2+} loading (Fig. 8F, red vs. blue). Red model simulations predict that I_f amplitude at MDP decreases by 44%, i.e., similar to the experimental results (Fig. 8, red trace), but the steady state AP firing rate decreases by only 7% (Fig. 8, red trace), i.e., about half of the experimentally measured steady state AP firing rate.

cAMP-PKA and CaMKII dependent signaling both affect phosphorylation of PLB, which reduces SERCA2 Ca^{2+} pumping by shifting the half maximal $[\text{Ca}^{2+}]$ for Ca^{2+} uptake. The model takes into account the effects of cAMP and CaMKII signaling on the Ca^{2+} clock in the presence of IVA by changing the value of K_{up} (Half-maximal Ca_i for Ca^{2+} uptake in the network SR). Specifically, K_{up} was changed to fit the experimentally measured decrease in SR Ca^{2+} load. The model simulation in which the crosstalk of the Ca^{2+} clock by the M clock

occurs in the presence of decreased PLB phosphorylation (green color), not only reproduced the experimentally measured reduction in I_f during diastolic depolarization (Fig. 8), but also reproduced the full experimentally measured reduction of the spontaneous AP firing rate in response to IVA (Fig. 8, green trace). Although, as in the red trace the peak I_{CaL} only slightly decreases (by 2%), the I_{CaL} integral per 10 sec decreases from 194 in the absence of drugs to 167 pA*s. In this model simulation, when both clocks can fully entrain each other, IVA further prolonged the cytosolic Ca^{2+} transient T-90_c (by 15%), substantially decreases maximal Ca_{jSr} (by 7%) and maximal Ca_{nsr} (by 12%), in accord with the experimental observations (Fig. 8 and Table S5). Moreover, this model predicted that, on the basis of its effect to entrain the LCR characteristics, IVA shifted the timing at which inward sarolemmal Na^+ - Ca^{2+} exchanger current was activated by Ca^{2+} at a later time during diastolic depolarization (by 14%) (Fig. 8G), i.e. which is precisely the same extent to which IVA reduced the AP firing rate.

The green tracings in Fig. 8 show the model simulations in which crosstalk to the Ca^{2+} clock by the M clock also occurs, due to a reduction in PLB phosphorylation (reduction in K_{up}) in response to IVA. Note that when this additional crosstalk is introduced into the model (i.e., reduction in PLB phosphorylation) the I_{NCX} integral during diastolic depolarization becomes further reduced, to 187 pA*mV. In both red and green simulations, changes in steady state I_{NCX} are related to the changes in steady state spontaneous AP firing rate (Fig. 9). Therefore, these model simulations suggest that IVA leads to an **indirect** effect to reduce I_{NCX} during late diastolic depolarization. Moreover, although the reduction in I_{NCX} is less in the model simulations when IVA only induced a reduction in I_f than in the model simulation that includes a reduction in PLB phosphorylation, the change in the I_f integral (from 30 to 19 pA*mV) during diastolic depolarization (−65 to −45 mV) was similar in both simulations.

4. Discussion

The first novel finding of our work is that IVA indirectly suppresses intracellular Ca^{2+} cycling at a concentration that specifically inhibits I_f but does not directly suppress intracellular Ca^{2+} cycling (i.e., via a direct effect on I_{CaL} [6, 18] or SR Ca^{2+} cycling (Fig. 7)), and does not affect other surface membrane ion channels (i.e., I_K or I_{Na}) [6, 19]. Specifically, our results demonstrate that although a direct effect on Ca^{2+} cycling and SR Ca^{2+} loading in response to IVA does not occur, SR Ca^{2+} load is reduced and spontaneous LCRs from SR via ryanodine receptors are suppressed. This can be explained on the level of a net reduction in Ca^{2+} influx associated with the reduced AP firing rate (due to reduced number of APs that occur per unit time). During transition to a new steady state, reduction in Ca^{2+} influx simultaneously leads to (1) a reduction in Ca^{2+} available for pumping into the SR, reducing the SR Ca^{2+} load (the prolongation of the cytosolic Ca^{2+} transient rate T-90_c can be interpreted to indicate a reduced rate of Ca^{2+} pumping into SR (Fig. S4) [13]), (2) prolongation of the LCR period and (3) a shift of Ca^{2+} activation of I_{NCX} to later time during diastolic depolarization. In other terms, intracellular Ca^{2+} cycling of the Ca^{2+} clock, informed by the feed-forward effect of the reduced AP firing rate to reduce Ca^{2+} influx, feeds back to entrain the M clock to further reduce the AP firing rate. As a result of the reduced net Ca^{2+} influx and SR Ca^{2+} load, a reduction of the amplitude of the LCR ensemble Ca^{2+} signal and prolongation of its period lead to a reduction and delayed in activation of I_{NCX} and also likely to reduced Ca^{2+} -calmodulin activation of AC-cAMP/PKA and CaMKII signaling axes (for review, see [20]). This reduction in phosphorylation of Ca^{2+} cycling proteins further reduces the net Ca^{2+} influx and SR Ca^{2+} load. A new steady-state equilibrium is achieved as Ca^{2+} efflux via I_{NCX} becomes sufficiently reduced commensurate with reduced Ca^{2+} influx due to the reduction in AP firing rate [21]. Note, that while 3 μ M IVA affects only I_f , higher concentrations of IVA (> 10 μ M) directly reduce I_{CaL} [6, 18] and would lead to direct reduction in intracellular Ca^{2+} . Therefore concentrations of IVA higher

than 3 μM cannot be used as a tool to define the cross talk between the membrane and Ca^{2+} clock.

Thus, we interpret the results of our IVA experiments to indicate that the magnitude of bradycardia in response to IVA is not determined solely by an effect produced by one channel, i.e., funny-current ion channel, but rather by potent crosstalk within a coupled-clock system. To test this interpretation, we employed CPA to directly and selectively inhibit SERCA2 Ca^{2+} pumping into the SR. Using this approach, we determine the magnitude of AP firing rate reduction in response to a reduction in SR Ca^{2+} load and suppression of LCR characteristics to levels that were similar in magnitude to those affected by IVA. These experiments demonstrated that CPA decreases the SR Ca^{2+} load, and prolonged the LCR period, without directly affecting $I_{\text{Ca,L}}$ or I_f (Figs. S2 and S3). Similar to the entrainment effect of the AP firing rate reduction by IVA on the Ca^{2+} clock, the reduction in AP firing rate in response to CPA involves crosstalk between M and Ca^{2+} clocks. Ca^{2+} pumping into SR via SERCA was directly suppressed by CPA, leading to a reduction in SR Ca^{2+} load, the LCR period is prolonged, and the spontaneous LCR ensemble Ca^{2+} signal to the M clock to activate NCX is reduced and delayed. The resultant Ca^{2+} to M clock crosstalk leads to a reduction of the AP firing rate. The AP rate reduction, per se, (during transition to a new steady state) provides a feed-forward effect to reduce net Ca^{2+} influx, and thus the resultant reduction in intracellular Ca^{2+} simultaneously feeds back on SR to further entrain reductions in SR Ca^{2+} cycling kinetics and prolongation of the LCR period. While we used only one concentration of IVA, by using CPA at a concentration that induces similar reduction in SR Ca^{2+} load, we show that **two** different perturbations that have completely different specific and direct mechanisms of actions induce a similar suppression of LCR characteristics and a similar reduction in magnitude of AP firing rate. The effects of CPA and IVA on coupled-clock entrainment are similar, even in the absence of an effect of CPA to reduce I_f or an effect of IVA to directly affect SR Ca^{2+} cycling. Indeed, when the shift in the relationship between the changes in LCR period and AP cycle length in response to reduction in I_f (IVA) or SERCA2 inhibition (CPA) are superimposed (Fig. 6), it becomes apparent that IVA and CPA effects on LCR period and cycle length are similar. This is the essence of our main hypothesis i.e., that crosstalk occurs between the membrane and Ca^{2+} clock when either the membrane or the Ca^{2+} clock is directly perturbed. Thus, it may be concluded that, no matter how it is perturbed, the LCR period reports the level of crosstalk within the coupled-clock system. In other words, our results demonstrate that the net change in AP cycle length evoked by selectively perturbing either the M clock or the Ca^{2+} clock is predictable by, and inseparable from, the concurrent change in LCR periodicity and amplitude, and therefore LCR characteristics report an integrated function (i.e., coupling or entrainment) of M and Ca^{2+} clocks.

Because the additive contributions of the feed-back mechanisms from the entrained clock (indirectly perturbed) to the perturbed clock, the contributors to the steady state reduction in spontaneous AP firing rate cannot be determined experimentally (Fig. 8–9). We approached their dissection *in silico*, in the context of a numerical model. Model simulations that include only limited crosstalk between the M and Ca^{2+} clocks (i.e., due to the reduction in Ca^{2+} influx that inevitably occurs when the AP firing rate is reduced) in the absence of effects of the change in intracellular Ca^{2+} to reduce the extent of Ca^{2+} -calmodulin activation of AC-cAMP/PKA and CaMKII-dependent phosphorylation of PLB fail to reproduce the experimental results. In other terms, the model simulation in the absence of additional crosstalk that results from a reduction in Ca^{2+} cycling protein phosphorylation (i.e., PLB) can account for only about 50% of the experimentally measured reduction in steady state AP firing rate that occurs in response to IVA. In contrast, simulations in which the model is informed of reductions in Ca^{2+} -calmodulin PKA and CaMKII phosphorylation-dependent PLB signaling predict the full experimentally observed reduction in steady state AP firing

rate induced by IVA. These model simulations, therefore, strongly suggest that both feed-back and feed-forward mechanisms resulting from a reduction in Ca^{2+} -calmodulin activation of AC-cAMP/PKA and/or CaMKII signaling axes lead to a reduction in Ca^{2+} cycling protein phosphorylation that contributes importantly to the overall effect of IVA to reduce the steady state AP firing rate. Specifically, the model simulations predict that a reduction in the spontaneous AP firing rate results in reduction in Ca^{2+} influx and to a suppression of SR Ca^{2+} cycling which induces a reduction in cAMP/PKA and CaMKII signaling axis, leading to a reduction of PLB phosphorylation which leads to a further reduction in SR Ca^{2+} cycling and AP firing rate. Note that, while a reduction in Ca^{2+} activation of AC to reduce cAMP may shift the steady-state activation of I_f this could also contribute to IVA-induced change in the AP firing rate. Our model simulations, however, predicted that this effect is likely to be minor, because a maximum shift in I_f activation (e.g. up to ~ 8 mV in response to acetylcholine [22]) results only in a minor decrease of the AP firing rate (within 4%, not shown).

When our model (Fig. 8 and 9) includes only limited crosstalk between the membrane and Ca^{2+} clocks (in the absence of effects of the change in intracellular Ca^{2+} due to the reduction in AP firing rate to reduce the extent of Ca^{2+} -calmodulin activation of AC-cAMP/PKA and CaMKII-dependent phosphorylation of PLB), the model fails to reproduce the steady-state changes in spontaneous AP firing rate by IVA. Our model parameters of I_f prior to and during application of IVA are those that have been measured in the present study (Table S1d). It is interesting to note that another model, in which I_f amplitude is larger than our model [23], can even with limited crosstalk between the membrane and Ca^{2+} clocks reproduce the changes in spontaneous AP firing rate in response to IVA that were observed in the experiments of the present study. This computational model, however in contrast to our experimental results, predicts only a modest decrease in SR Ca^{2+} load, resulting from changes in maximal Ca_{jsr} (by 3%) and maximal Ca_{nsr} (by 4%). Therefore, this computational model predicts that changes in steady state spontaneous AP firing rate by IVA are mainly due to changes in membrane clock (I_f), and not due to crosstalk between the clocks. This result is not in accord with experimental results.

The experimental effects of IVA to reduce the SANC AP firing rate are mediated only in part by its direct effects to inhibit I_f and in part, by its crosstalk with the Ca^{2+} clock (Fig. 3–7), indicating that the SANC AP firing rate response to perturbations is regulated by a coupled-clock system, not by a single effector molecule alone, or by a single clock. Thus, our results support the hypothesis that crosstalk between membrane and Ca^{2+} clocks regulates SANC normal automaticity. The steady-state response to a perturbing signal that directly and specifically perturbs only one clock is determined by both feed-forward and feed-back effects between the clocks, which determine the magnitude of change in AP firing rate. Our combined experimental data and numerical model simulations support the idea that the effect of IVA to reduce the steady-state SANC AP firing rate involves continual crosstalk between the Ca^{2+} and M clocks during the transition of the AP firing rate from its initial level to steady-state level: a net reduction in Ca^{2+} influx during this transition is linked to instantaneous reduction of the AP firing rate and reduced Ca^{2+} influx per unit time. Intracellular Ca^{2+} signaling is suppressed in response to the reduction in Ca^{2+} influx and Ca^{2+} available for pumping into the SR is reduced. Thus, the reduced SR Ca^{2+} load leads to a reduction in the LCR ensemble and prolongation of its period, which causes a reduction in magnitude and a shift of Ca^{2+} activation of I_{NCX} to a later time during diastolic depolarization. Intracellular Ca^{2+} reduction leads to reduction in Ca^{2+} -calmodulin activation of the adenylyl cyclase-cAMP-PKA and CaMKII signaling axis. This amplifies the feed-forward effect of the AP rate to reduce the SR Ca^{2+} pumping, SR Ca^{2+} load and Ca^{2+} influx. A new steady-state equilibrium is achieved as Ca^{2+} efflux via I_{NCX} becomes reduced to

balance reduced Ca^{2+} influx initially due to the reduction in AP firing rate that later is amplified by phosphorylation-dependent signaling.

Thus, under voltage clamp even though a low dose of IVA may specifically inhibit I_f , experiments of the present study in isolated SANC suggest that the interpretation that the beneficial heart rate reduction by IVA involved in recent clinical trials (BEAUTIFUL, SHIFT, VIVIFY, etc. [1]) does not occur **solely** on the basis of direct I_f inhibition. The demonstration that crosstalk between the clocks determines the steady rate reduction in the response to IVA does not diminish its clinical therapeutic efficacy to reduce heart rate, but informs a more correct interpretation of why the heart rate becomes reduced to the extent that it does in response to IVA. Thus, our experiments and theoretical modeling that support such crosstalk between the membrane and Ca^{2+} clock suggest that application of IVA, together with a drug that affects the Ca^{2+} clock, may increase the beneficial effect of IVA to reduce heart rate. Alternatively, additive effects of the concurrent use of IVA and drugs that reduce cell Ca^{2+} cycling may induce extended rate reduction. Additional experiments are required, however, to determine the extent to which the mutual clock entrainment observed in SANC in response to IVA in the present study occurs in the intact heart/organism.

5. Limitations

We isolate the sinoatrial node tissue utilizing the anatomical landmarks within the right atrium area. Following disaggregation of the sinoatrial node to obtain single cells it is not possible to determine the original location of an isolated cell within the intact node prior the isolation. Some studies have found the cells within the SAN center differ from those in SAN periphery [24] other studies that demonstrate that some peripheral cells (and atrial cells as well) penetrate the central SAN, suggest that there may not be a homogenous cell population within the central area [25]. Although the basal spontaneous AP firing rate and AP characteristics of rabbit isolated SAN cells do not differ between central and peripheral cells (based upon cell size) [26], the funny channel density does differ between small and larger cells [24]. Therefore, IVA may indeed have different effects on different cells in our study that cannot be distinguished using the isolation landmarks in the present study.

Change in PKA or CaMKII-dependent protein phosphorylation signaling affects other protein than PLB phosphorylation, e.g., L-type channel, K^+ channels and ryanodine receptors [27]. However, due to technical issues that prevent measurements of other phosphorylation sites there is a lack of sufficient experimental data to quantify these effects in pacemaker cells. PLB phosphorylation, on the other hand, has been measured with other drug intervention [11] and has been employed in numerical models as a general index of changes in protein phosphorylation, i.e., not only of PLB, but also of other protein that were not measure experimentally. Due to direct interaction of Ca^{2+} influx and SR Ca^{2+} load, *Kup* is changed in the model to simulate a change in PLB phosphorylation that results in a change in SR Ca^{2+} load. Although changes in other phosphorylations sites surly occur, in the model changes in PLB phosphorylation are sufficient to correctly simulate the experimental results of changes in SR Ca^{2+} load and spontaneous AP firing rate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations and acronyms

AC	Adenylyl-cyclases
AP	Action potential
CPA	Cyclopiazonic acid
IVA	Ivabradine
LCR	Local Ca^{2+} release
M	Membrane
MDP	Maximum diastolic depolarization
PKA	Protein kinase A
PLB	Phospholamban
SANC	Sinoatrial-node cells
SR	Sarcoplasmic reticulum
T-50_c	50% decay time of intracellular Ca^{2+}
T-90_c	90% decay time of intracellular Ca^{2+}

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Highlights

- Bradycardic effects of ivabradine include reduction in intracellular Ca^{2+} cycling.
- Local Ca^{2+} release period reports the crosstalk between membrane and Ca^{2+} clocks.
- Definitive evidence for a coupled-clock function in heart pacemaker cells.
- Possible clinical mechanism for ivabradine induced bradycardia.

Figure 1. Schematic illustrations of the coupled clock system

The interplay of Ca^{2+} -calmodulin adenylyl-cyclases (AC), PDE activity and cAMP-mediated, protein kinase A (PKA)-dependent and Ca^{2+} /calmodulin protein kinase II (CaMKII) signaling to the cardiac pacemaker sarcoplasmic reticulum Ca^{2+} cycling proteins, surface membrane ion channels and mitochondria.

Figure 2. Effect of IVA on I_f amplitude and activation kinetics

(A) Representative I_f traces in SANC recorded before and 5 min after application of IVA and (B) average peak I_f amplitude-voltage relationship (inset depicts I_f measured over the physiological voltage range), (C) representative I_f tail traces recorded in SANC before and after application of IVA and (D) I_f steady-state activation curve under control conditions, and in the presence of IVA (n=9). I_f tail current density at each membrane potential in control and with IVA is expressed relative to its maximal value at -120 mV.

Figure 3. Effect of IVA on spontaneous AP firing rate and spatiotemporal characteristics of LCRs

(A) Representative AP recordings and (B) average changes in the rate of AP firing in the presence of IVA (n=9). (C) Confocal line scan images of Ca^{2+} in a representative SANC before and following exposure to IVA. LCRs are indicated by arrowheads. The LCR period is defined as the time from the peak of the prior AP-induced Ca^{2+} transient to an LCR onset. (D) LCR size (full width at half-maximum amplitude) (n=12 cells, 123 LCR) and (E) A linear function describes the relationship of the 90% decay time of the AP-induced Ca^{2+} transient (T-90c) to the LCR period in control and in the presence of IVA. The line is the best least squares linear-regression fit to the data. *p<0.05 vs. drug.

Figure 4. SR load estimation from rapid caffeine application

Effects of a rapid application of caffeine (indicated by the arrow) onto a SANC in (A) control, or in the presence of $3 \mu\text{M}$ IVA. (B) Average effects of IVA on the amplitude of caffeine-induced cytosolic Ca^{2+} transient ($n=12$ in control and in response to IVA). Effects of a rapid application of caffeine onto a SANC in (C) control, or in the presence of CPA. (D) Average effects of CPA on the amplitude of the caffeine-induced cytosolic Ca^{2+} transient ($n=9$ for control and CPA). (Note that the caffeine response can be usually measured only once in a given SANC, because following caffeine application a prolonged period is required for AP firing rate to return to the control AP firing rate. Therefore, the effects of caffeine before (i.e., control) and following application of IVA are measured in different cells.) * $p<0.05$ vs. control.

Figure 5. Effect of CPA on spontaneous AP firing rate and spatiotemporal characteristics of LCRs

(A) Ca^{2+} confocal line scan images of a representative SANC before and following exposure to CPA, (B) LCR size in control ($n=12$ cells, 117 LCRs) and the presence of CPA ($n=12$ cells, 86 LCRs). (C) A linear function describes the relationship of the 90% decay time of the AP-induced Ca^{2+} transient (T-90c) to the LCR period in control and in the presence of CPA. The line is the best least squares linear-regression fit to the data. (D) Representative AP recordings and (E) average changes in the rate of AP firing in the presence of CPA ($n=9$). * $p<0.05$ vs. drug.

Figure 6. Effect of IVA and CPA on LCR period

The LCR period in control or in the presence of IVA (panel A) or CPA (panel B) predicts the concurrent AP cycle length. (C) Combined data in A and B for IVA and CPA conform to a least square fit of the data in A and B for IVA or CPA alone, respectively. Inset illustrates the average data points for control, IVA and CPA. (D) Data in panel C for IVA or CPA are expressed as % change from control prior to drug application in each cell. The IVA and CPA prolongation of the LCR period predict the concurrent prolongation of AP cycle length. The line is the best least squares linear-regression fit to the data. The dash line is the line of identity.

Figure 7. Effect of IVA on spontaneous LCR characteristics in saponin permeabilized SANC
 (A) Ca^{2+} confocal line scan images of a representative SANC before and following exposure to IVA. Average effects of IVA on the (B) LCR duration, (C) LCR size and (D) LCR amplitude.

Figure 8. Coupled clock numerical model

Simulations of the effects of IVA on (A) I_f , (B) AP, (C) I_{CaL} , (D) cytosolic Ca^{2+} , (E) junctional, (F) network SR Ca^{2+} and (G) sarolemmal Na^+-Ca^{2+} exchanger current. Blue traces are model simulations prior to drug. Red traces are model simulations when feed-forward entrainment and with only partial feed-back between the M and Ca^{2+} clock. Green traces are model simulations when both feed-forward and full feed-back entrainments exist between the clocks. Note the dashed line represent the diastolic depolarization phase.

Figure 9.

Numerical simulations of the relationships between membrane potential, I_f and sodium calcium exchange current during (A) and (C) one complete AP cycle; (B) and (D) during diastolic depolarization only. Note, that the ordinate scale in A and C differs from that in B and D.