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The Role of the Calcium and the Voltage Clocks in Sinoatrial Node Dysfunction

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Recent evidence indicates that the voltage clock (cyclic activation and deactivation of membrane ion channels) and Ca^{2+} clocks (rhythmic spontaneous sarcoplasmic reticulum Ca^{2+} release) jointly regulate sinoatrial node (SAN) automaticity. However, the relative importance of the voltage clock and Ca^{2+} clock for pacemaking was not revealed in sick sinus syndrome. Previously, we mapped the intracellular calcium (Ca_i) and membrane potentials of the normal intact SAN simultaneously using optical mapping in Langendorff-perfused canine right atrium. We demonstrated that the sinus rate increased and the leading pacemaker shifted to the superior SAN with robust late diastolic Ca_i elevation (LDCAE) during β-adrenergic stimulation. We also showed that the LDCAE was caused by spontaneous diastolic sarcoplasmic reticulum (SR) Ca^{2+} release and was closely related to heart rate changes. In contrast, in pacing induced canine atrial fibrillation and SAN dysfunction models, Ca^{2+} clock of SAN was unresponsiveness to β-adrenergic stimulation and caffeine. Ryanodine receptor 2 (RyR2) in SAN was down-regulated. Using the prolonged low dose isoproterenol together with funny current block, we produced a tachybradycardia model. In this model, chronically elevated sympathetic tone results in abnormal pacemaking hierarchy in the right atrium, including suppression of the superior SAN and enhanced pacemaking from ectopic sites. Finally, if the LDCAE was too small to trigger an action potential, then it induced only delayed afterdepolarization (DAD)-like diastolic depolarization (DD). The failure of DAD-like DD to consistently trigger a sinus beat is a novel mechanism of atrial arrhythmogenesis. We conclude that dysfunction of both the Ca^{2+} clock and the voltage clock are important in sick sinus syndrome.

Key Words: Calcium, sinoatrial node, sarcoplasmic reticulum, sick sinus syndrome

INTRODUCTION

The sinoatrial node (SAN) automaticity is responsible for initiating the heart rhythm. SAN function is therefore essential for normal cardiac physiology. Although it has been shown more than 40 years ago that spontaneous diastolic depo-

larization of SAN cells periodically initiates action potentials to set the rhythm of the heart, the mechanism of heart rhythm generation is still unclear. Sick sinus syndrome is an abnormality involving the generation of the action potential by the SAN and is characterized by an atrial rate inappropriate for physiological requirements. The sick sinus syndrome occurs in 1 of every 600 cardiac patients older than 65 years and accounts for approximately half of implantations of pacemakers in the United States.[1] A better understanding of the mechanisms of SAN automaticity and sick sinus syndrome is therefore clinically important.

Voltage and calcium clocks in the SAN automaticity

The mechanism of spontaneous diastolic depolarization has traditionally been attributed to a "voltage clock" mechanism, mediated by voltage-sensitive membrane currents, such as the hyperpolarization-activated pacemaker current (I_f) regulated by cyclic adenosine monophosphate (cAMP).[2,3] The funny channel becomes activated at the end of the action potential (at voltages from - 40/- 50 mV to - 100/- 110 mV), which corresponds to the range where diastolic depolarization occurs. It then depolarizes the membrane to a level where L-type Ca^{2+} channel open to initiate the action potential.[4,5] I_f is a mixed Na^+-K^+ inward current activated by hyperpolarization and modulated by the autonomic nervous system. Because the membrane ionic channels open and close according to the membrane potential, this process is referred to as membrane voltage clock. The major role of I_f has been reinforced by the fact that mutations in the I_f channel are associated with reduced baseline heart rate,[6] and drugs which blocks I_f (such as ivabradine) do the same.[7] However, while point mutation of hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) is associated with baseline sinus bradycardia, the maximum heart rate achieved during exercise is normal.[8] The latter finding implies that I_f is not the only mechanism of SAN automaticity, especially during sympathetic activation.

Recently, the spontaneous sarcoplasmic reticulum (SR) Ca^{2+} release was suggested as an additional mechanism of sinus rhythm generation also known as the Ca^{2+} clock. The involvement of intracellular calcium (Ca_i) cycling in heart rhythm generation was first suggested from the observation that application of ryanodine slowed subsidiary pacemakers in cat right atrium.[9] Subsequent work showed the involvement of the electrogenic Na-Ca exchange current (I_{NCX}) in pacemaker activity and also raised the possibility that increased Ca^{2+} release followed by activation of I_{NCX} could play a role in the positive chronotropic effect of β-adrenergic stimulation in latent pacemaker cells.[10-12] Lakatta, et al. suggested that the spontaneous rhythmic SR Ca^{2+} release in SAN cells, manifested as Ca^{2+} sparks, work as the "Ca^{2+} clock". The elevated Ca_i causes diastolic depolarization via I_{NCX} activation, which coordinately regulates sinus rate along with the voltage clock.[13-15]

The idea of Ca^{2+} clock suggests that the mechanism of automaticity is the same as that of delayed afterdepolarization (DAD), which occurs when there is SR Ca^{2+} overload. This suggests that the SAN must exist normally in a state of calcium overload. Vinogradova, et al.[15] also demonstrated that a high basal level of protein kinase A (PKA) activity in the SAN might contribute to a state of Ca^{2+} overload. The disease associated with Ca^{2+} clock malfunction was also reported in patients with a genomic deletion of ryanodine receptor 2 (RyR2) exon-3. Patients with that mutation develop catecholaminergic polymorphic ventricular tachycardia, along with SAN and atrioventricular node dysfunction, atrial fibrillation and atrial standstill.[16] It is possible that Ca^{2+} clock malfunction contribute to the bradycardia and atrial arrhythmias in these patients.

Pacemaker hierarchy and the importance of Ca^{2+} clock in intact SAN

The cardiac automaticity at the organ level is a very complex phenomenon and, beside cellular mechanisms, integrative factors are involved in cardiac pacemaking. The intact SAN is a heterogeneous structure that includes multiple different cell types interacting with each other.[17-19] The relative importance of the voltage and Ca^{2+} clocks for pacemaking in different regions of the SAN, and in response to neurohumeral stimuli such as β-agonists, may be different. Indeed, activation maps in intact canine right atrium (RA) showed that SAN impulse origin is multicentric,[20] and sympathetic stimulation predictably results in a cranial (superior) shift of the pacemaking site in human and dogs.[21,22] Based on evidence from isolated SAN myocytes, late diastolic Ca_i elevation (LDCAE) relative to the action potential upstroke is a key signature of pacemaking by the Ca^{2+} clock.[10-15] It is possible that the same phenomenon could provide insights into the relative importance of the Ca^{2+} and voltage clock mechanisms in pacemaking in intact SAN tissue. We therefore designed a study to determine the role of Ca^{2+} and voltages clocks on the heart rhythm generation and on the mechanisms of pacemaker hierarchy in the intact SAN.[23]

Heterogeneous Ca_i dynamics in intact SAN

At baseline, the spontaneous diastolic SR Ca^{2+} release, which is manifested by the LDCAE, was observed in only a small percentage of the preparations. However, LDCAE occurred in all preparations during isoproterenol infusion, associated with a superior shift of the leading pacemaker site, coincident with the appearance of robust LDCAEs (Fig. 1) in this region. Most importantly, the site of maximum LDCAE slope always co-localized with the leading pacemaking site, suggesting a shift in which the voltage clock now lagged the Ca^{2+} clock (Fig. 2). This observation indicates a strong association between LDCAE and pacemaking during β-adrenergic stimulation, and provides new insights into pacemaker hierarchy in the canine RA.[20-22]

The Ca_i Dynamics of SAN were characterized not only by the earliest onset of LDCAE but also by the fastest Ca_i reuptake as compared with other RA sites. The baseline 90% Ca_i relaxation time was shorter at superior SAN than at other RA sites. This resulted in the formation of the Ca_i sinkhole, which was facilitated by a rapid decline (short relaxation time) of the Ca_i fluorescence at the superior SAN during isoproterenol infusion and suggests that Ca_i reuptake by SR is the fastest in the superior SAN (Fig. 1D). The key protein regulator of SR Ca^{2+} uptake is phospholamban, which inhibits SERCA2a in its dephosphorylated state. There was a significantly lower SERCA2a/phospholamban ratio at SAN sites than at RA sites, suggesting more phospholamban molecules are available to regulate SERCA2a molecules in SAN than in RA. Isoproterenol infusion phosphorylates phospholamban and relieves phospholamban inhibition of SERCA2a, which may account for more robust Ca^{2+} uptake in SAN than in RA during isoproterenol infusion.[23]

Mechanisms of diastolic depolarization of SAN

The LDCAE was closely related with the SR Ca^{2+} release. Caffeine sensitizes the ryanodine receptor 2 to activation, resulting in increased SR Ca^{2+} release.[24] The superior shift of LDCAE and the pacemaking site was also consistently observed with caffeine infusion. The ryanodine, which block ryanodine receptors, caused a dose-dependent suppression of sinus node activity, and impaired isoproterenol-

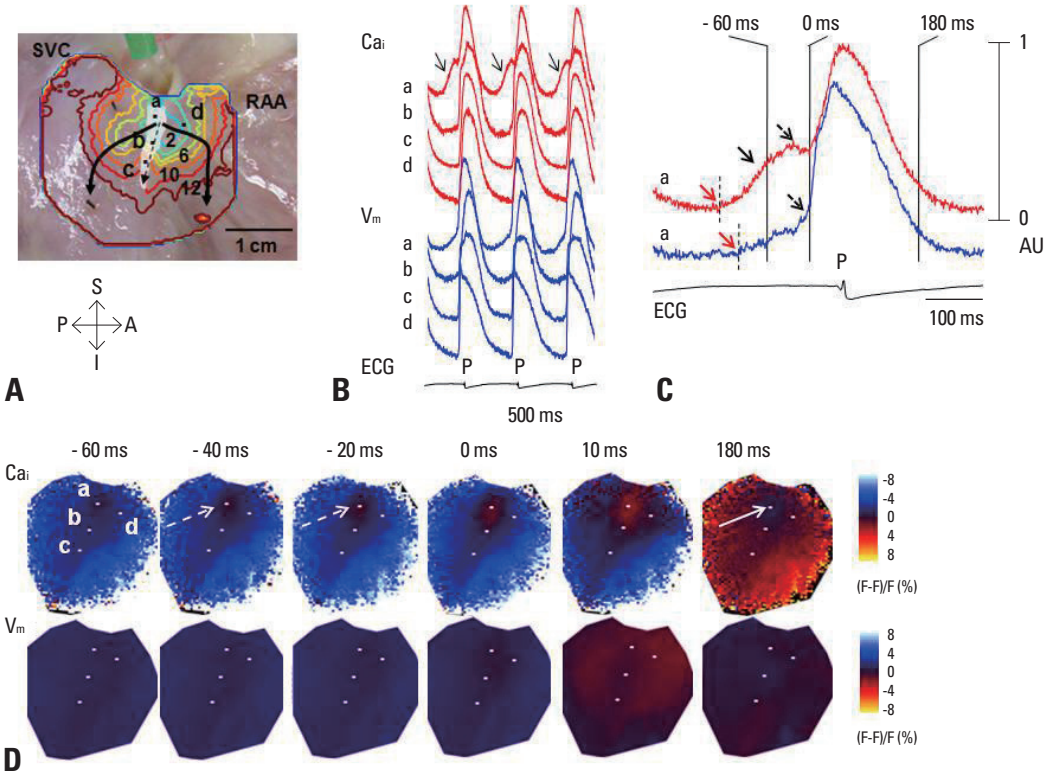

Fig. 1. Activation pattern of SAN and surrounding RA during isoproterenol infusion of 0.3 μmol/L. (A) Isochronal map of V_m. The number on the each isochronal line indicates time (ms). White shaded area is the SAN. (B) The V_m (blue) and Ca_i (red) recordings from the superior (a), middle (b), inferior (c) SANs and RA (d) presented in A. (C) Magnified view of Ca_i and V_m tracings of superior SAN. Note the robust LDCAE (solid arrow) before phase 0 of action potential (0 ms), which in turn was much earlier than onset of p wave on ECG. (D) The V_m and Ca_i ratio maps at times from - 60 ms before to 180 ms after phase 0 action potential of C. The LDCAE (broken arrows in frame - 40 and - 20 ms) was followed by the Ca_i sinkhole during early diastole (solid arrow in frame 180 ms). This figure was reproduced with permission from Joung, et al.[23] SAN, sinoatrial node; RA, right atrium; LDCAE, late diastolic Ca_i elevation.

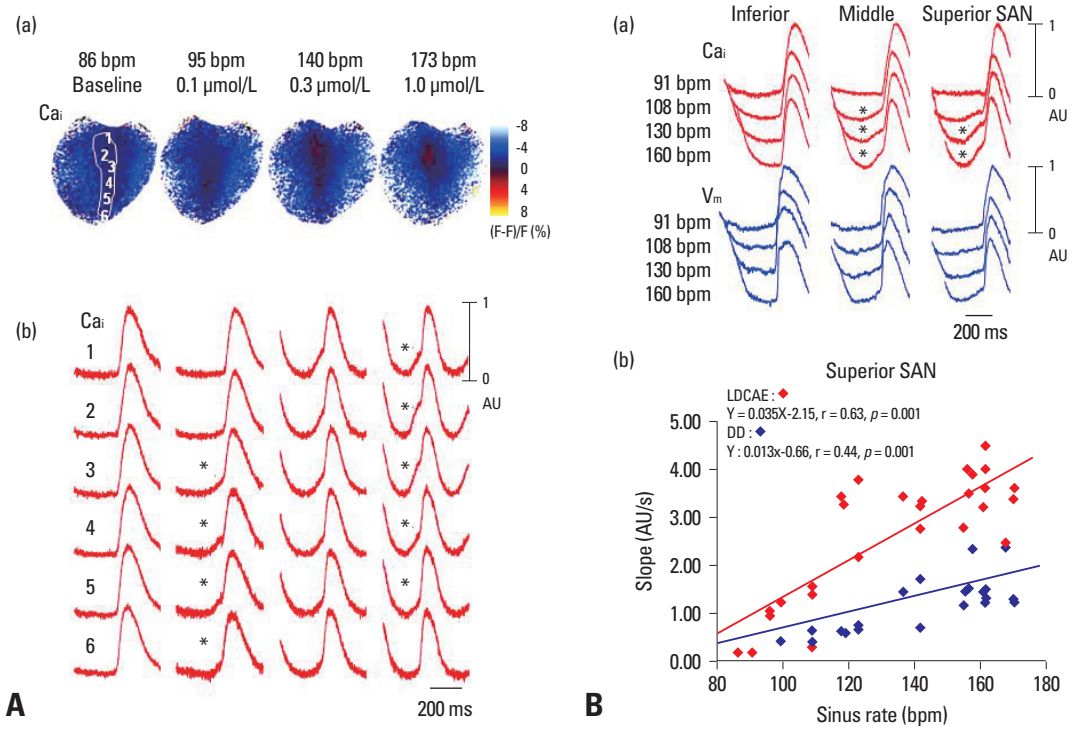

Fig. 2. Co-localization of LDCAE and the leading pacemaker site. (A) Upward shift of the leading pacemaker site with LDCAE during isoproterenol infusion. (a) Ca_i ratio maps of SAN at each sinus rate. (b) Corresponding Ca_i tracings from superior (1, 2), middle (3, 4) and inferior (5, 6) SAN. At 95 bpm, the sites 4 and 5 had most prominent LDCAEs (asterisks). As sinus rate gradually increased, the sites of Ca_i elevation progressively moved upward. At the maximum sinus rate of 173 bpm, the site 2 had the most apparent LDCAE. (B) Differential responses of different SAN sites to isoproterenol. (a) The Ca_i and V_m tracings from inferior, middle, and superior SAN sites at different sinus rates. (b) The LDCAE and DD slopes of superior SAN at different sinus rates. This figure was reproduced with permission from Joung, et al.[23] SAN, sinoatrial node; LDCAE, late diastolic Ca_i elevation; DD, diastolic depolarization.

induced LDCAE. The combination of ryanodine and thapsigargin also suppressed the sinus node activity, and impaired isoproterenol-induced LDCAE. In contrast, the I_f blocker, ZD 7288 (3 μmol/L) did not prevent LDCAE in the superior SAN.

Multiple time- and voltage-dependent ionic currents have been identified in cardiac pacemaker cells which contribute to diastolic depolarization, including $I_{Ca\text{-}L}$, $I_{Ca\text{-}T}$, I_{ST} and various types of delayed rectifier K currents.[25] Many of these membrane currents are known to respond to β-adrenergic stimulation. Some of these currents, such as $I_{Ca\text{-}L}$, also promote LDCAE and the acceleration of sinus rate by the Ca^{2+} clock as well as the voltage clock. In intact SAN, both SR inhibitors and I_f blockade slowed sinus rate under basal conditions, as well as blunted the isoproterenol-induced increase in sinus rate. Therefore, the interdependence and synergy between the two clocks are evident.

Impaired Ca^{2+} clock after β-adrenergic stimulation in AF dogs

Atrial fibrillation (AF)-induced remodeling of ionic currents has been well documented in the atrium.[26,27] The typical electrophysiological remodeling in AF includes action potential duration (APD) shortening, the downregulation of L-type Ca^{2+} channel ($I_{Ca\text{-}L}$) caused by atrial cardiomyocyte Ca^{2+} loading,[28,29] downregulation of I_{to}[30] and upregulation of IKACh and IK1.[26,27] The ionic current remodeling could reduce the slope of phase 0, hyperpolarize the Vm and reduce the heart rate. However, the mechanism of tachycardia induced-SAN dysfunction is unclear. Yeh, et al.[31] recently reported that I_f downregulation may contribute to the association between SAN dysfunction and supraventricular tachyarrhythmias. However, normal functioning SAN depends not only on membrane ionic currents but also on the rhythmic Ca^{2+} releases from the SR.[10-15]

In a recent study, we found that the SAN dysfunction in AF is associated with Ca^{2+} clock malfunction, characterized by unresponsiveness to isoproterenol and caffeine, as well as downregulation of RyR2 in SAN. Fig. 3A shows a typical isoproterenol response of RAs from normal dogs. Isoproterenol infusion increased heart rate to and shifted the leading pacemaker site to superior SAN with a robust LDCAE [arrows in Ca_i tracing of Fig. 3A(b)]. This finding was consistently observed in all normal RAs during isoprotere-

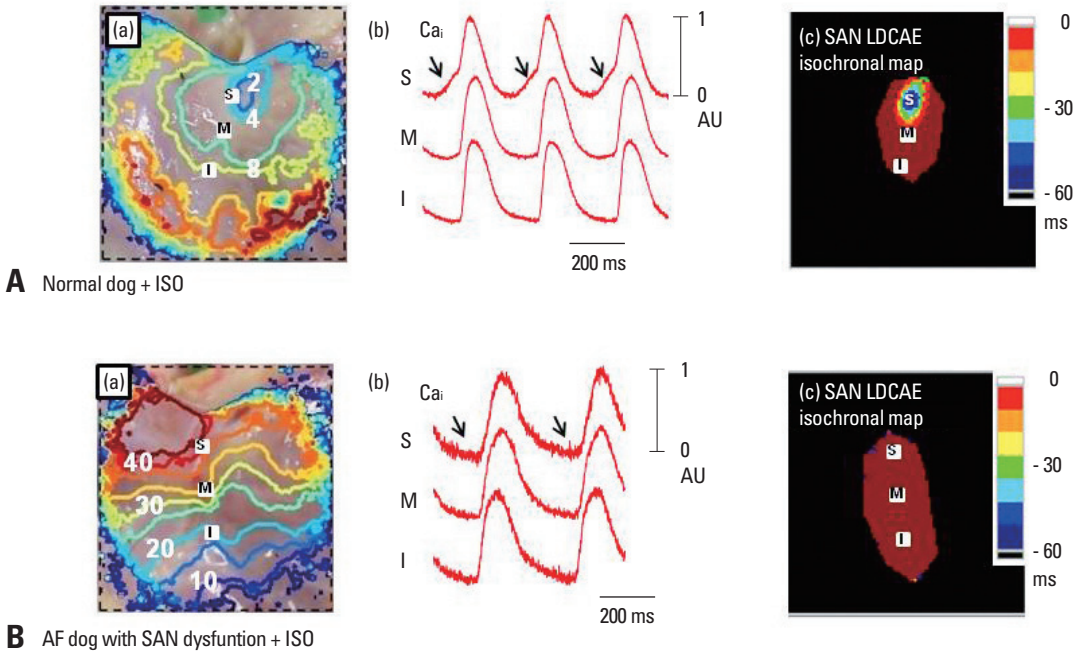

Fig. 3. Complete absence of LDCAE in AF dogs during isoproterenol infusion. (A) Isoproterenol response of normal dogs. In normal dogs, isoproterenol increased the heart rate and shifted the leading pacemaker site to the superior SAN with robust LDCAE (arrows). (B) Isoproterenol response of AF dogs. The LDCAE was complete absent in AF dogs during isoproterenol infusion. (a) RA V_m isochronal map. (b) Ca_i tracings from superior (S), mid (M), and inferior (I) SANs. (c) SAN LDCAE isochronal map. The unit of numbers on RA V_m isochronal map is ms. The earliest activation of RA was considered as 0 ms. This figure was reproduced with permission from Joung, et al.[32,33] SAN, sinoatrial node; AF, atrial fibrillation; RA, right atrium; LDCAE, late diastolic Ca_i elevation.

nol infusion. However, LDCAE increase in superior SAN was completely absent in AF dogs [Fig. 3B(b)]. Also, the heart rate was increased by the acceleration of the ectopic focus from inferior RA [Fig. 3B(a)]. The Ca_i tracing showed no LDCAE anywhere in the mapped region with isoproterenol dose ranging from 0.01 to 10 μmol/L.[32,33]

Sympathetic stimulation and tachybradycardia syndrome

It is known that heart failure is frequently associated with SAN remodeling, resulting in decreased SAN reserve.[34] We performed nerve recording in a canine model of pacing-induced heart failure and found intermittent tachybradycardia episodes.[35] Interestingly, the prolonged (>3 s) sinus pauses were triggered not by vagal activation but by short bursts of sympathetic activity. Typically, a burst of sympathetic activity is associated with tachycardia. When there is sympathetic withdrawal, the tachycardia terminates, followed by prolonged pauses during which no activation was observed. The molecular mechanism of this association, however, remains unclear.

In a recent study, we developed various model of sick sinus syndrome with pharmacological manipulation of Ca^{2+} and membrane ion clock. Combined malfunction of both membrane and Ca^{2+} clocks underlie the mechanisms of long sinus pauses. Prolonged (- 1 hour) isoproterenol infusion simulates the persistently elevated sympathetic tone, which is typical in patients with heart failure but can also occur in normal individuals. The persistently elevated sympathetic tone by itself does not induce tachybradycardia. However, I_f blockade in the presence of prolonged sympathetic stimulation could produce tachybradycardia (Fig. 4). This finding highlights the importance of I_f current in maintaining normal SAN function in conditions with persistently increased sympathetic tone, such as heart failure. Similar to the experimental condition, important hallmarks of heart failure include both chronically elevated sympathetic tone[35] and a concomitant reduction of I_f.[36] The chronically increased sympathetic tone is known to have profound effects on the cardiac contractile function and arrhythmogenesis.[37] However, the effects of chronic prolonged catecholamine stimulation on SAN remains poorly understood.

Our study was the first to map Ca^{2+} clock function in the SAN during prolonged isoproterenol infusion. LDCAE slope and 90% Ca_i relaxation time reached peak at 5 ± 2 min after isoproterenol infusion, and decreased after prolonged infusion. This finding occurred with the shift of the leading pacemaker site from superior to inferior SAN (Fig. 5).

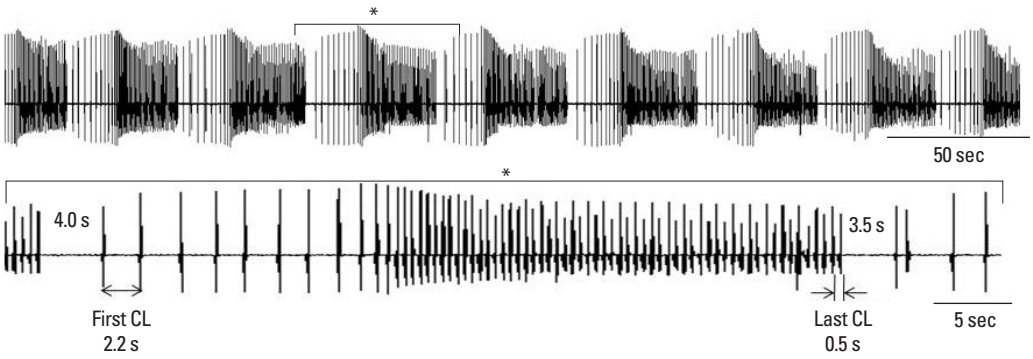

Fig. 4. Tachybradycardia produced by prolonged isoproterenol and ZD 7288 infusion. The figure shows pseudo ECG from canine isolated RA. Upper panel, eight episodes of tachybradycardia. Lower panel, expanded view of a section marked by asterisk (*) showing 4.0 s and 3.5 s pauses. This figure was reproduced with permission from Joung, et al.[39] RA, right atrium.

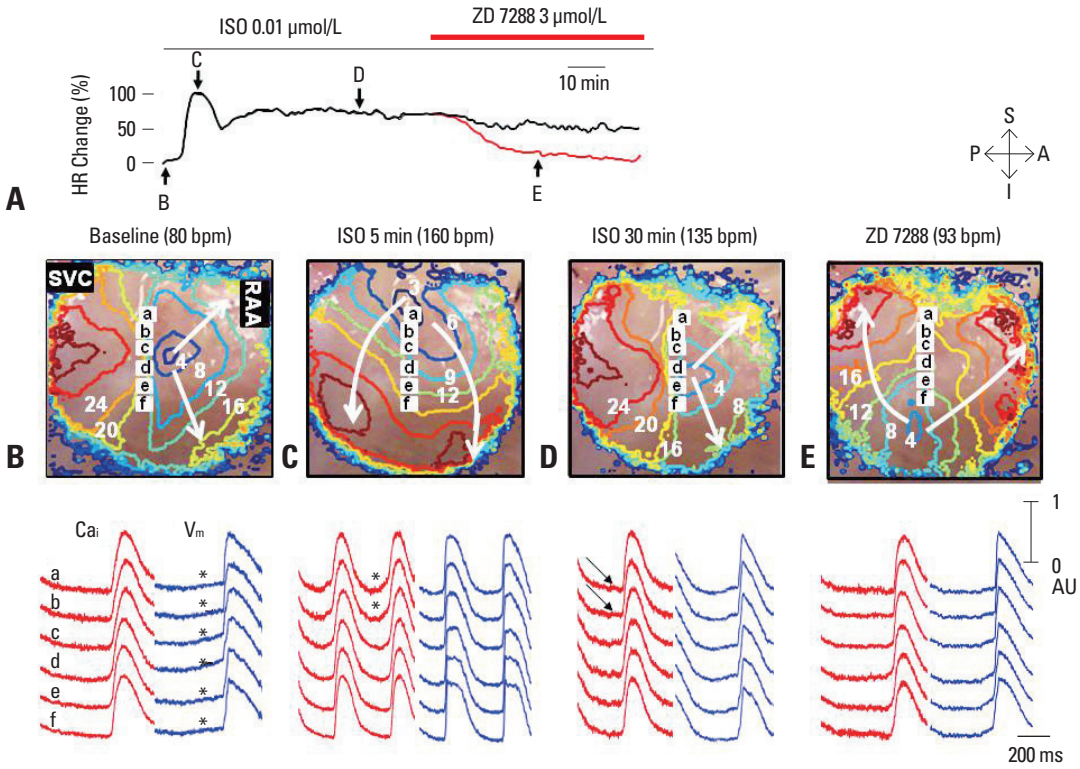

Fig. 5. The effect of prolonged low dose isoproterenol infusion (n = 8). (A) Heart rate trends after prolonged (> 1 hr) isoproterenol infusion of 0.01 μmol/L. Heart rate was increased maximum at 5 min, and decreased after 10 min. ZD 7288 infusion of 3 μmol/L decrease the heart rate again almost to the baseline level. B through E show V_m isochronal map of SAN and surrounding RA (upper panels), and Ca_i (red) and V_m (blue) tracings from superior (a and b), middle (c and d) and lower (e and f) SAN. (B) Baseline. Note the diastolic depolarization (asterisks) in SAN. C, Isoproterenol infusion for 5 min. Note the LDCAE (asterisks) from superior SAN. (D) Isoproterenol infusion for 1 hr. Note the disappearance of LDCAE with shifting of the leading pacemaker site to inferior SAN (site e). (E) ZD 7288 infusion for 30 min. The pacemaker site shifted to the more inferior SAN (site f). The unit of numbers on V_m isochronal map is ms. This figure was reproduced with permission from Joung, et al.[39] SAN, sinoatrial node; LDCAE, late diastolic Ca_i elevation.

SNRT and cSNRT were increased after ZD 7288 infusion in the presence of prolonged isoproterenol infusion. These findings provide new insights into the mechanism of SAN dysfunction commonly found in patients with heart failure.[38]

Our results from an *in vitro* tachybradycardia model indicate that chronically elevated sympathetic tone results in abnormal pacemaking hierarchy in the RA, including suppression of the superior SAN and enhanced pacemaking from ectopic sites.[39]

Subthreshold DAD and a new mechanism of atrial arrhythmia

Automaticity and triggered activity are thought to be two distinct mechanisms for the initiation of heart beats. Auto-

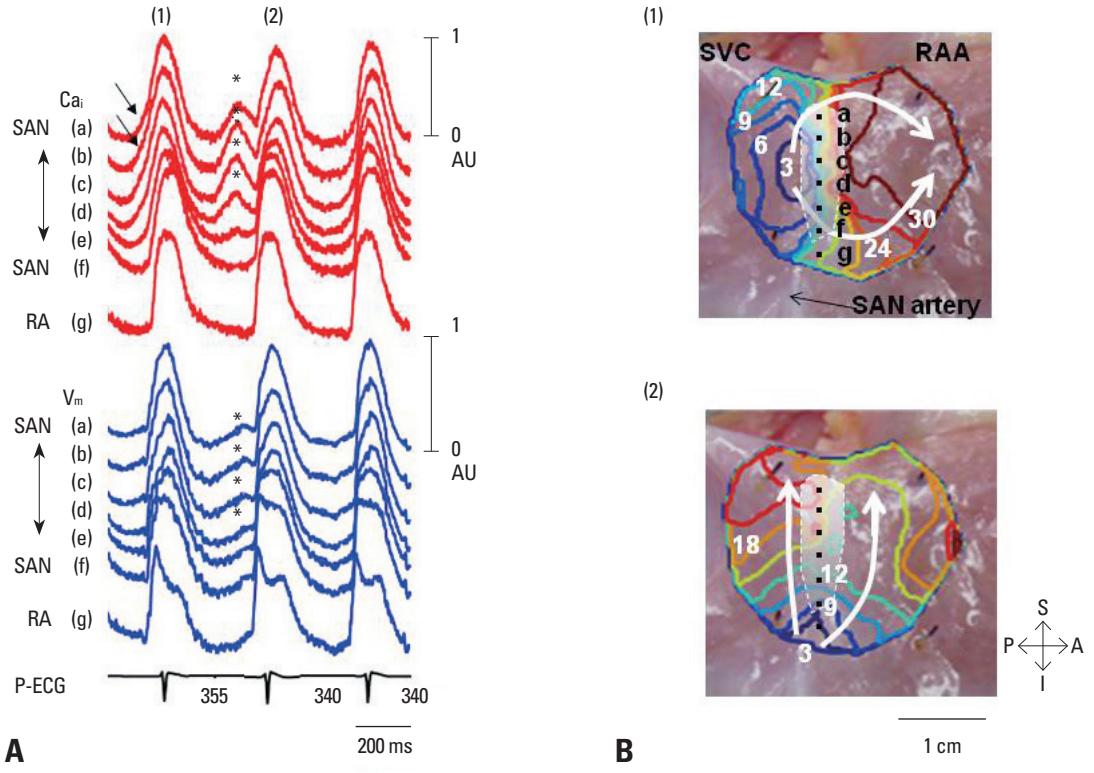

Fig. 6. The intermittent pattern of subthreshold DADs. These tracings were obtained during isoproterenol infusion (0.03 μmol/L) in the first RA preparation. (A) Optical signals of Ca_i (red) and V_m (blue) from superior (a and b), middle (c and d), and inferior (e and f) SAN, and RA (g). There were 3 consecutive activations in this figure. Among them, the first 1) and third beats show LDCAE (arrows) followed by the initiation of sinus beats from the same sites. In contrast, the second beat 2) showed both LDCAE on Ca_i tracings and subthreshold DADs on V_m tracings (asterisks). The downslope of the subthreshold DADs were observed because they failed to trigger an action potential. (B) V_m isochronal maps of the first (1) and second (2) beats. The white shaded area is the SAN. The first beat 1) was from SAN. Because subsequent LDCAE in the SAN (asterisks in Panel A) failed to trigger a sinus beat, an ectopic pacemaker was able to take over and activate the mapped region 2). This figure was reproduced with permission from Joung, et al.[51] RAA, right atrial appendage; SVC, superior vena cava; A, anterior; P, posterior; S, superior; I, inferior, DAD, delayed afterdepolarization; SAN, sinoatrial node; RA, right atrium; LDCAE, late diastolic Ca_i elevation.

maticity occurs spontaneously and can be a source of both normal and abnormal heart beats, while triggered activity is pacing-induced and is almost always pathological. A mechanism of triggered activity is spontaneous (non-voltage gated) sarcoplasmic reticulum (SR) Ca release, which causes Na-Ca exchanger current (I_{NCX}) activation and membrane depolarization, resulting in delayed afterdepolarization (DAD).[40] When DAD reaches threshold, it initiates triggered activity and arrhythmia (reverse excitation-contraction coupling).[41,42] Recent studies, however, showed that rhythmic spontaneous Ca release ("Ca clock")[43-45] may work together with hyperpolarization-activated membrane currents ("membrane clock") to generate normal sinus rhythm, a prototypical example of normal automaticity. These findings suggest that SAN activity may share mechanisms that underlie both automaticity and triggered activity, i.e., I_{NCX} activation.[11,12,14,40,46-49] Consistent with this hypothesis, Bogdanov, et al.[49] showed that in single isolated SAN cells, spontaneous SR Ca release in conditions of impaired I_{NCX} may result in membrane potential (V_m) oscillation without leading to regenerative action potential. However, whether or not DADs can occur in the intact SAN remains unknown.

We demonstrated that in intact RA preparation, failure of subthreshold DAD to reach threshold allowed latent pacemakers elsewhere to activate the atrium, resulting in atrial arrhythmia (Fig. 6). In these arrhythmic episodes, a beat that closed the longer PP interval, rather than a premature beat, was from an ectopic focus. This phenomenon was also compatible with the concept of parasystole in which the SAN was a source of normal rhythm while the ectopic pacemaker was the parasystolic focus. When the SAN failed to generated a rhythm to inhibit (or pre-excite) the parasystolic focus, the latter was able to exit and capture the entire RA. Shinohara, et al.[50] recently used the same intact RA preparation to study the mechanisms of pacemaking of the

ectopic pacemakers. They found that while spontaneous SR Ca release underlies isoproterenol-induced increase of superior SAN activity, the atrial ectopic pacemaker is less dependent on the Ca clock and more dependent on the membrane clock for its automaticity. These ectopic pacemakers outside the SAN therefore can effectively serve as backup pacemakers when SAN fails. The co-existence of two pacemaking sites resulted in atrial arrhythmias observed in the present study.

CONCLUSIONS

The voltage and Ca^{2+} clocks jointly regulate SAN automaticity. In various models of sick sinus syndrome, the dysfunction of both clocks was consistently observed. Our results from normal and pathological SANs strongly support the notion that the Ca^{2+} clock and the voltage clocks work synergistically to generate SAN automaticity.

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