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Review

Dynamic interactions of an intracellular Ca^{2+} clock and membrane ion channel clock underlie robust initiation and regulation of cardiac pacemaker function

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For almost half a century it has been thought that the initiation of each heartbeat is driven by surface membrane voltage-gated ion channels (M clocks) within sinoatrial nodal cells. It has also been assumed that pacemaker cell automaticity is initiated at the maximum diastolic potential (MDP). Recent experimental evidence based on confocal cell imaging and supported by numerical modelling, however, shows that initiation of cardiac impulse is a more complex phenomenon and involves yet another clock that resides under the sarcolemma. This clock is the sarcoplasmic reticulum (SR): it generates spontaneous, but precisely timed, rhythmic, submembrane, local Ca^{2+} releases (LCR) that appear not at the MDP but during the late, diastolic depolarization (DD). The Ca^{2+} clock and M clock dynamically interact, defining a novel paradigm of robust cardiac pacemaker function and regulation. Rhythmic LCRs during the late DD activate inward Na^{+}/Ca^{2+} exchanger currents and ignite action potentials, which in turn induce Ca^{2+} transients and SR depletions, resetting the Ca^{2+} clock. Both basal and reserve protein kinase A-dependent phosphorylation of Ca^{2+} cycling proteins control the speed and amplitude of SR Ca^{2+} cycling to regulate the beating rate by strongly coupled Ca^{2+} and M clocks.

1. The concept of membrane ion channel 'clocks' in cardiac pacemaker cells

The sinoatrial nodal pacemaker cells (SANC) normally 'autoexcite'. Their rhythmic action potentials (APs) are preceded by a slow diastolic depolarization (DD) which brings the membrane potential to an excitation threshold. The classical point of view[1–3] portrays the entire duty cycle of pacemaker cells as a simple reciprocal activation of surface membrane voltage-gated ion currents, comprising a self-sustained membrane oscillator or a 'membrane clock' (M clock) (*Figure 1A*). According to Hodgkin–Huxley formalism,[4] the ion channels are opened (or activated) by a change in membrane voltage and undergo a time-dependent transition into an inactivated state. Thus, kinetics of activation and inactivation of the channels underlie the timing mechanisms of the currents that comprise the M clock (*Figure 1B*, 'Currents'):

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1.1 Action potential

Voltage-gated Na^{+} current (I_{Na}) is absent in cells of the primary pacemaker region of the sinus node, and the AP upstroke is mainly formed by activation of L-type Ca^{2+} current (I_{CaL}) in these cells. The depolarization activates outward K^{+} currents, which eventually prevail over inward currents and repolarize the membrane towards its lowest level within the cycle, the maximum diastolic potential (MDP). The MDP of about -65 mV is higher than the resting potential of ~ -90 mV in ventricular myocytes or the MDP in Purkinje fibres ranging from -70 to -85 mV, because SANC expresses either no inward rectifier K^{+} current (I_{K1}) or, in some species, a very small I_{K1}.[5]

1.2 The early diastolic depolarization

The 'g_K decay'[6] is the major mechanism of the early DD in SANC.[7] Following the achievement of the MDP, the conductance for delayed rectifier K^{+} current (I_K) decreases as the K^{+} channels inactivate after their activation during AP. This shifts the balance in favour of inward currents resulting

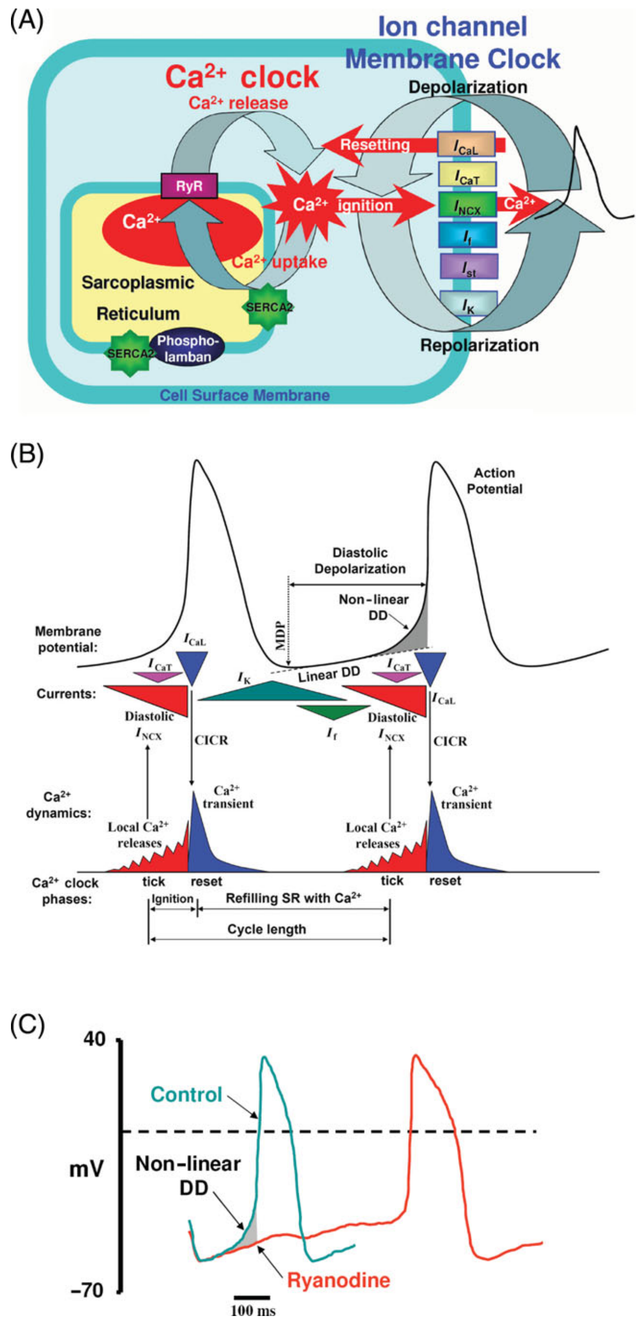

Figure 1 (*A*) A schematic illustration of a novel pacemaker mechanism for the sinoatrial node cell. A classic membrane ion channel clock operates not in isolation, but in tight cooperation with yet another clock (Ca^{2+} clock) located under cell membrane. The dynamically coupled clocks produce robust heartbeats, as Ca^{2+} clock ignites membrane excitations but membrane clock (M clock) resets the Ca^{2+} clock each pacemaker cycle via Ca^{2+}-induced Ca^{2+} release (CICR). (*B*) A schematic illustration of the key phases of the interactions between M clock (membrane potential and ion currents) and Ca^{2+} clock (Ca^{2+} dynamics and Ca^{2+} clock phases) comprising the pacemaker mechanism (modified from[72]). (*C*) Ryanodine abolishes the Ca^{2+} clock-mediated action potentials ignition by inhibition of the non-linear diastolic depolarization (DD) (grey area shown by arrow). Modified from[37].

in a depolarization immediately after the MDP. This response is essentially an afterpotential (or afterhyperpolarization), which is also observed in other cardiac cell types.[8] The repolarization during the later part of the AP, also activates another early-mid DD mechanism, the hyperpolarization-activated 'funny' current (I_f).[9,10] While, in the past, this current has been referred to 'the pacemaker current', it is not the dominant pacemaker mechanism in SANC.[1,11,12] Experimental blockade of I_f results only in a minor increase of the cycle length (5–20%) in SANC. Due to its low activation voltage (< -65 mV) and slow activation kinetics, I_f is responsible for a slower DD in the pacemaker range of < -65 mV of subsidiary pacemaker cells rather than of SANC.[7]

The late diastolic depolarization

The contribution of I_{CaL} activation to DD has been suggested as early as in 1980 (I_s at that time).[13] The threshold of I_{CaL} was found to be 'positive from -45 to -50 mV',[10] 'around -60 mV',[14] or 'between -50 and -40 mV'.[15] Taken these varying estimates into account, and also that steady-state activation curve for I_{CaL} in rabbit SANC initiates at -50 mV,[16] I_{CaL} likely becomes activated at -50 mV. Such a relatively low activation threshold of I_{CaL} in SANC might be explained, at least in part, by two factors: (i) An experimental evidence suggests that L-type Ca^{2+} channels could be phosphorylated by highly active protein kinase A (PKA) in the basal state;[17,18] (ii) a low activation threshold L-type Ca^{2+} channel isoform $Ca_v1.3$ as shown in mouse SANC[19] might also contribute to I_{CaL} in rabbit SANC. Activation of T-type Ca^{2+} current (I_{CaT}) was suggested as an additional mechanism contributing to the mid-late DD: Since this current has a low activation threshold (~ -60 mV), its activation might trigger a depolarization leading to activation of the conventional I_{CaL}.[20] Finally, a non-selective sustained current (I_{st})[21] was suggested as yet another DD mechanism. However, the identity of I_{st} remains still unclear, because it exhibits many properties of I_{CaL} and Na^+/Ca^{2+} exchanger (NCX) current (discussed later), and its molecular origin and specific blockers have not been found.

2. Theoretical modelling of the membrane clock: limitations and previous attempts of integration with intracellular processes

The understanding of complexity of cardiac pacemaker function is traditionally approached by using theoretical modelling. A most recent review by Wilders[3] discusses no fewer than 12 different SANC models that have been published since 1980. Despite an apparent triumph of the application of the Hodgkin–Huxley formalism to describe the shape of AP and DD of SANC, a unique, definitive formulation has not been found. The models react differently to the perturbations (e.g. ion channel blockade), indicating that the simple membrane-delimited approach to describe SANC operation has fundamental and technical limitations.

2.1 Multiple current components, species- and cell population- dependency

I_K was resolved into rapid and slow components, I_{Kr} and I_{Ks}, respectively. Expression of many currents turned out to be species dependent. For example, bullfrog sinus venosus cells lack I_f.[22] An I_{K1} of small amplitude is present in mouse, rat, and monkey; I_{Ks} is present in porcine SANC, but I_{Kr} operates in rat and rabbit (under basal conditions).[5] I_{CaT} likely contributes to pacemaker function in relatively small animals having high basal beating rate (e.g. in mouse or rat), but its contribution decreases as the animal size increases.[5] For example, I_{CaT} makes a minor contribution to rabbit SANC function, and similar to blockade of I_f, blockade of I_{CaT} results in a minor cycle length increase of $\sim$14[23] or $\sim$16%.[24] I_{CaT} is absent in porcine SANC.[25] I_{Na} and chloride current (I_{Cl}) are observed only in subpopulations of SANC.[26] I_{CaT} is detected in only 20% of rabbit SANC (in five from 25 cells tested).[15]

2.2 An extremely tiny, delicate balance of multiple factors underlies the diastolic depolarization time course

Because of high membrane resistance during DD, a net ion current change driving the DD has an extremely small scale ($\sim$3 pA in rabbit SANC).[10] On the other hand, ion channels operate not in isolation, but within a tight heterogeneous environment that influences the channel function. For example, the channel gating properties can be modulated by ions, cAMP, phosphorylation, and interactions with other proteins. Furthermore, ion currents are produced not only by ion channels but also by electrogenic ion pumps and exchangers. Finally, SANC, like other cardiac cells, have an active sarcoplasmic reticulum (SR), the major Ca^{2+} store which can release Ca^{2+} and thereby influence both ion channels and transporters. Thus, any, even relatively weak, intracellular chemical signals, not described by classical M clock models, can easily influence the DD's fate. Further, measurement of characteristics of ion channels of the M clock require specific experimental conditions, in which the intracellular milieu is often perturbed by the method of measurement. When such data, obtained in the context of disrupted physiologic links, are incorporated into pacemaker models, the nature of the net current change in the spontaneously beating cell in the models may be different from that in rhythmically firing intact SANC.

2.3 Large spread in experimental data

Because of technical difficulties of the patch clamp technique, and because of widely varying recording conditions, the experimental data available for numerical modelling exhibit large variations (even within species, which traditionally has been rabbit). SANC modellers, therefore, have had a large degree of freedom with respect to which specific values of parameters (e.g. specific ion conductances) to employ in their formulations. Accordingly, a comparison of different pacemaker models[3] reveals that different currents drive the same tiny critical net ion current change during DD in different SANC models. As a result, all models generate rhythmic APs but their responses to perturbations greatly vary.

2.4 Previous attempts to integrate ion homeostasis and Ca^{2+} release with M clock

The fundamental limitations of the membrane-delimited approach have been realized long ago.[1] When formulations for ion transporters and Ca^{2+} release have been included into SANC models, the revised models better approximated cell ion homeostasis. However, Ca^{2+} release formulations in these models were adopted from atrial or ventricular cell models, because of the absence of Ca^{2+} measurement in SANC at the time. Those formulations closely predict a Ca^{2+} transient initiated by the AP,[27] but this Ca^{2+} transient has almost no contribution to the DD. Zhang *et al*.[28] has even excluded Ca^{2+} release formulations from their model and set again all ion concentrations to constant values because in their opinion 'inclusion of intracellular Na^+ and Ca^{2+} handling in the models is not essential'. Simulations of a more recent model[29] showed that while bulk cytosol Ca^{2+} transient decay continues for some time during the early DD, the Ca^{2+} transient in submembrane space ends sharply at the MDP (*Figure 5* in[29]). Since Ca^{2+} interacts with surface membrane proteins in submembrane space, the model predicts no contribution of the AP-induced Ca^{2+} transient into the early DD. This also indicates that measurements of submembrane Ca^{2+} dynamics are critical for instructive modelling of pacemaker activity.

3. Cardiac sarcoplasmic reticulum is yet another physiological clock

While 12 SANC models published since 1980[3] portray an essentially membrane-delimited SANC function, evidence that an intracellular mechanism is implicated in the initiation of the cardiac impulse stems from the turn of the last century (see[8] for review). Multiple, subsequent clues, i.e. spontaneous, oscillatory Ca^{2+}-driven events, confirmed the existence of an intracellular clock[1,8,30–32] within Purkinje fibres and the sinus node. However, conditions required to generate detectable spontaneous Ca^{2+} oscillations in pacemakers were grossly unphysiological (e.g. high [Ca^{2+}], low [Na^+], high or low [K^+] or cardiac glycosides). In the final analysis, the role of spontaneous intracellular Ca^{2+} releases was relegated solely to 'abnormal' pacemaker function or 'abnormal automaticity' (see[33] for review). For example, a Purkinje fibre model manifests repetitive APs evoked by spontaneous Ca^{2+} releases via activation of NCX current under Na^+/Ca^{2+} overload (the required $[Na^+]_i$ is 12 mM).[34] In increased $[K^+]_o$ membrane potential of SANC exhibits two types of oscillations: oscillatory afterpotential (V_{os}) and prepotential (ThV_{os}).[35,36] It turned out that V_{os} is induced by spontaneous SR Ca^{2+} release and can be abolished by caffeine, whereas ThV_{os} is produced by ion channels.[35]

However, extensive evidence had accumulated that cardiac SR is yet another *physiological* clock in cardiac cells (reviews[33,37,38]): SR can spontaneously cycle Ca^{2+} under physiological conditions and in cardiac pacemaker cells these spontaneous, rhythmic, intracellular Ca^{2+} releases are critically important to initiate normal automaticity. Indeed, cardiac SR has a Ca^{2+} pump and a Ca^{2+} release channel (ryanodine receptor, RyR) and the potential for spontaneous Ca^{2+} release is inherent in the design of this organelle. Isolated cardiac SR vesicles,[39] cardiac cell fragments in which the sarcolemma had been mechanically removed,[40,41] but SR function preserved, and electrochemically shunted cardiac myocytes[42] all exhibit such spontaneous SR Ca^{2+} releases.

When disconnected from the surface membrane, the SR of intact cardiac ventricular cells is no longer entrained by rhythmic APs and becomes 'free running': it generates spontaneous, roughly periodic Ca^{2+} releases, even in the context of physiologic intracellular [Ca^{2+}].[43] In contrast to the global 'systolic,' SR Ca^{2+} release into the cytosol triggered by an

AP, spontaneous SR Ca^{2+} release occurs locally within cells. Variable types of spontaneous, SR-generated, local Ca^{2+} releases, i.e. LCRs, exhibit varying degrees of synchronization, ranging from Ca^{2+} sparks,[44] to Ca^{2+} wavelets[18,24,45,46] or Ca^{2+} waves, which are more synchronized, and are roughly periodic. It is important to note that while a Ca^{2+} wave may travel the length of the cell, depleting the entire SR cell throughout, at any given instant, the wave-related Ca^{2+} release occurs within a relatively small part of the cell, that is, in the form of LCRs.[47,48]

The LCR period, i.e. the time required for spontaneous SR Ca^{2+} release to occur following a prior release, reflects the speed at which the Ca^{2+} clock 'ticks' in a given condition.[49,50] Since spontaneous SR-generated Ca^{2+} releases in ventricular cells are thought to occur when luminal SR Ca^{2+} achieves a threshold level,[49,51] their frequency is determined, at least in part, by how fast the SR can reload with Ca^{2+}. This depends upon how much Ca^{2+} is available for pumping, and the speed of the SR Ca^{2+} pump. The occurrence and characteristics of LCRs is cell type and species specific in a given condition.[52,53] Interventions effecting an increase in cell and SR Ca^{2+} loading, i.e. β-adrenergic receptor (β-AR) stimulation, an increase in bathing [Ca^{2+}], or cardiac glycosides, reduce the restitution time, and increase the frequency of successive SR Ca^{2+} releases. The frequency range of spontaneous SR Ca^{2+} releases, <0.1 to about 10 Hz, brackets the physiologic frequencies at which the heart can beat.[43]

4. The SR Ca^{2+} clock 'ticks' shortly before AP occurrence in sinoatrial nodal pacemaker cells

Fluorescence imaging of intracellular Ca^{2+} in rabbit SANC over the last decade has documented the occurrence of an not only AP-induced global cytosolic Ca^{2+} transients,[27] but also the occurrence of LCRs beneath the cell surface membrane during late DD of spontaneously firing SANC in the absence of Ca^{2+} overload (*Figure 1B*, 'Ca^{2+} dynamics'). Such LCRs occur in pacemaker cells in the form of multiple locally propagating wavelets beneath the cell membrane,[45,54] larger than the spontaneous Ca^{2+} sparks in ventricular cells, but markedly less than well organized Ca^{2+} waves that can propagate the length of ventricular cells.[43] Both the AP-induced Ca^{2+} transient and spontaneous LCRs have similar characteristics in both smaller and larger SANC.[55] In spontaneously firing rabbit SANC LCR occurrence during the late spontaneous DD does not require triggering by the depolarization.[46] LCRs can occur spontaneously in these cells in the absence of membrane potential changes: in voltage-clamped SANC, persistent, rhythmic LCRs generate rhythmic current fluctuations with the same periodicity; both periodic LCRs and current fluctuations are abolished by ryanodine.[37] Similar persistent LCRs occur in 'skinned' SANC, bathed in 100 nM [Ca^{2+}].[18,46]

While, basal, diastolic Ca^{2+} levels are low (∼150 nM) and do not differ in rabbit SANC and ventricular cells[46,56] the basal state SR Ca^{2+} clock within SANC restitutes and generates LCRs with a LCR period (<0.5 s), which is substantially less than in ventricular myocytes under basal conditions (∼10 s).[57] A recent discovery provides the clue as to why SANC SR Ca^{2+} clock speed is so rapid and why LCRs larger than Ca^{2+} spark: levels of cAMP and cAMP-dependent phosphorylation within SANC are high in the absence of β-AR stimulation.[18] In turn, basal cAMP levels within SANC are high due to high constitutive adenylyl cyclase activity.[18] Ca^{2+}-activated adenylyl cyclase isoforms, highly expressed in the brain, are expressed in rabbit[58,59] and guinea pig[60] SANC. Interestingly, basal phosphodiesterase (PDE) activity also appears to be markedly elevated in SANC,[61] likely as a mechanism to keep basal cAMP levels in check. High levels of constitutive A cyclase and PDE activity enable the SANC to rapidly respond to demands for a change in the cAMP level and cycle length.

Among proteins highly phosphorylated in the basal state are phospholamban, RyRs;[18] and indirect evidence suggests that L-type Ca^{2+} channels are likely to be phosphorylated in basal state as well.[17] PKA-dependent phosphorylation of these proteins presents the SR with an increased Ca^{2+} to be pumped (Ca^{2+} influx via I_{CaL}), speeds up the SR Ca^{2+} filling rate (phospholamban), and likely alters threshold for spontaneous Ca^{2+} release activation (RyR). The net effects of these phosphorylations create conditions that are required for spontaneous occurrence of the Ca^{2+} wavelet type LCRs (rather than smaller, non-propagating releases, Ca^{2+} sparks) during the DD of spontaneously firing SANC in the basal state.

5. Dynamic interaction of Ca^{2+} and membrane clocks. I: local Ca^{2+} releases ignite action potentials via synchronous activation of local inward Na^+/Ca^{2+} exchanger currents and late diastolic depolarization acceleration

The remainder of our review puts forth an hypothesis that the two clocks, M clock (section 1) and Ca^{2+} clock, do not just coexist in SANC, but rather dynamically interact during each cycle (vertical arrows between 'Ca^{2+} dynamics' and 'Currents' in *Figure 1B*), and that the system of the coupled clocks determines a novel mechanism of robust initiation and rate regulation of heartbeats.

Interfering with SR Ca^{2+} cycling disengages interactions of M clock with Ca^{2+} clock and thus unmasks intrinsic characteristics of M clock. It turns out that the M clock cannot sustain its physiological function without its partner, Ca^{2+} clock (*Figure 1C*; *Figure 2A*, 'Ry', 'BAPTA', and 'Li^+'; *Figure 2B*). As early as in 1989 it was demonstrated that ryanodine (interfering with SR Ca^{2+} release) has a profound negative chronotropic effect on automaticity of subsidiary atrial pacemakers.[62] Analyses of the ryanodine effect on AP shape led to the suggestion that Ca^{2+} released from the SR contributes to DD, primarily during its later half, and plays a prominent role in bringing the late pacemaker potential to the AP activation threshold. The critical importance of Ca^{2+} cycling and NCX function for generation of spontaneous AP has been demonstrated in further studies employing ryanodine in various types of cardiac pacemaker cells of different species under normal conditions.[27,63–66] Although the magnitude of the ryanodine effect varies among intact sinoatrial node (SAN) preparations and isolated SANC, and varies among experimental studies, all studies have demonstrated a negative chronotropic effect of ryanodine (review).[67] Chelation of intracellular [Ca^{2+}] by fast Ca^{2+} buffers, e.g. BAPTA, also markedly attenuates or abolishes spontaneous beating in SANC.[16]

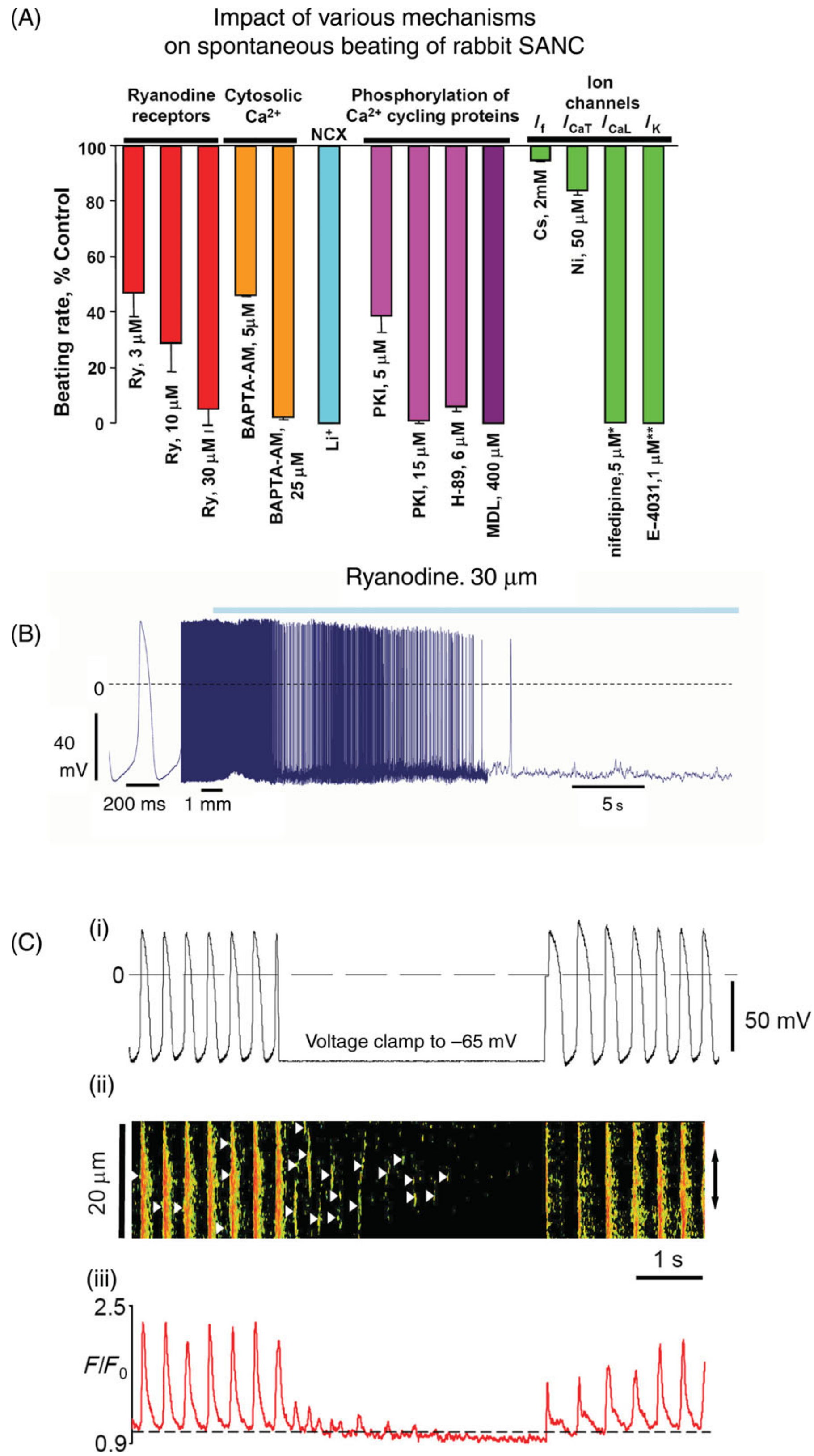

Figure 2 Interdependence of Ca^{2+} and membrane clocks (M clocks). (*A*) Spontaneous beating of rabbit sinoatrial nodal cells (SANC) critically depends upon both sarcoplasmic reticulum (SR) and membrane function as well as protein phosphorylation. Bars show a decrease in the beating rate (% control) induced by different drugs that affect Ca^{2+} cycling (ryanodine receptors, cytosolic Ca^{2+}), Na^+/Ca^{2+} exchanger (NCX) (Li^+ substitution for Na^+), protein phosphorylation (PKI, H-89, MDL), or ion channels: I_f (Cs^+), I_{CaT} (Ni^{2+}), I_{CaL} (nifedipine,*[14]), I_K (E-4031,**[92]). PKI and H-89 are PKA inhibitors, and MDL is an adenylyl cyclase inhibitor. Modified from[37]. (*B*) Inhibition of Ca^{2+} releases by ryanodine halts SANC automaticity (from[55]). (*C*) Ca^{2+} clock cannot sustain its long-term operation when M clock is inoperative in SANC under voltage clamp at the maximum diastolic potential (MDP). (i) and (ii) Simultaneous recordings of membrane potential and line-scan image of normalized subsarcolemmal fluo-3 fluorescence, respectively. LCRs are shown by white triangles; (iii) the time course of the normalized fluorescence averaged spatially over the band indicated by double headed arrow in (ii).

More recent studies in SANC, employing membrane potential recordings, combined with confocal Ca^{2+} measurements showed that LCRs impart a nonlinear, exponentially rising phase to the DD later part (*Figure 1B* and *C*).[45,68] Ion currents that generate this late DD acceleration can be inhibited by ryanodine or an NCX blockade.[45] Thus, the acceleration is determined, in large part, by an inward current (I_{NCX}) generated by electrogenic exchange of Ca^{2+} to 3 Na^{+} by NCX that is activated by LCRs, which begin to 'boil' and then 'explode' at this phase of pacemaker cycle.[45,68–70] While the effect of individual, stochastic LCRs on the DD is relatively small (~0.2 mV), a synchronized/cooperative action of multiple LCRs impart the exponentially rising phase to the DD and activate I_{CaL}.[68] A failure to generate diastolic I_{NCX} signals and an exponentially rising DD phase leads to pacemaker failure, even when Ca^{2+} influx via I_{CaL} is normal (under ryanodine or short-term NCX blockade)[45] or enhanced, e.g. in ischaemia-like conditions.[71,72]

The morphological background for this functional Ca^{2+} clock-NCX crosstalk has recently been demonstrated in rabbit SANC (*Figure 3*).[55] The average isolated SANC whole cell immuno-labelling density of RyRs and SERCA2 is similar to atrial and ventricle myocytes, and is similar among SANC of all sizes. Labelling of NCX1 is also similar among SANC of all sizes, and exceeds that in atrial and ventricle myocytes. Submembrane colocalization of NCX1 and cardiac RyR in all SANC exceeds that of the other cell types.[55] Furthermore, the Cx43 negative primary pacemaker area of the intact rabbit sinoatrial node (SAN) exhibits robust immuno-labelling for cardiac RyR, NCX1 and SERCA2.[55] Thus, there is a dense association of SERCA2, RyRs, and NCX1 in small-sized SANC, thought to reside within the SAN centre,[73] the site of SAN impulse initiation. The critical effects of Ca^{2+} chelation, NCX blockade and ryanodine on SANC function have been demonstrated in all SANC, independent of cell size.[55]

6. Dynamic interaction of Ca^{2+} and membrane clocks. II: surface membrane ion channels generate action potentials, reset the Ca^{2+} clock, and control cell Ca^{2+} balance

The powerful and sustained LCR signalling in SANC is achieved by the tight functional integration of SR and plasma membrane proteins. I_{CaL}-mediated Ca^{2+} influx during AP triggers CICR, i.e. a relatively synchronous SR Ca^{2+} release observed as a global Ca^{2+} transient.[27] This causes a global SR Ca^{2+} depletion that quenches the spontaneous LCRs in SANC and resets their local SR Ca^{2+} clocks. The resetting insures the functional integrity of the entire SR as one organelle: multiple, individual 'free running' local Ca^{2+} clocks of local segments of SR (having slightly different basal rates) generate synchronized LCRs later in diastole, resulting in powerful net Ca^{2+} signals. Therefore, I_{CaL} in resetting the Ca^{2+} clock, greatly amplifies LCR impact on the DD. The magnitude of L-type channel Ca^{2+} influx, itself, is finely tuned by the channel inactivation by the cytosolic Ca^{2+} transient that it triggers, and by high basal CaMKII-activity, which increases the number of available Ca^{2+} channels and accelerates their reactivation.[16] Since NCX function also depends on membrane voltage, NCX-mediated ignition of APs is tuned by characteristics of the afterpotential, i.e. the membrane voltage attained at the late DD due to I_K and I_f kinetic transitions in early and mid DD (section 1). Thus, rhythmic LCRs entrain rhythmic

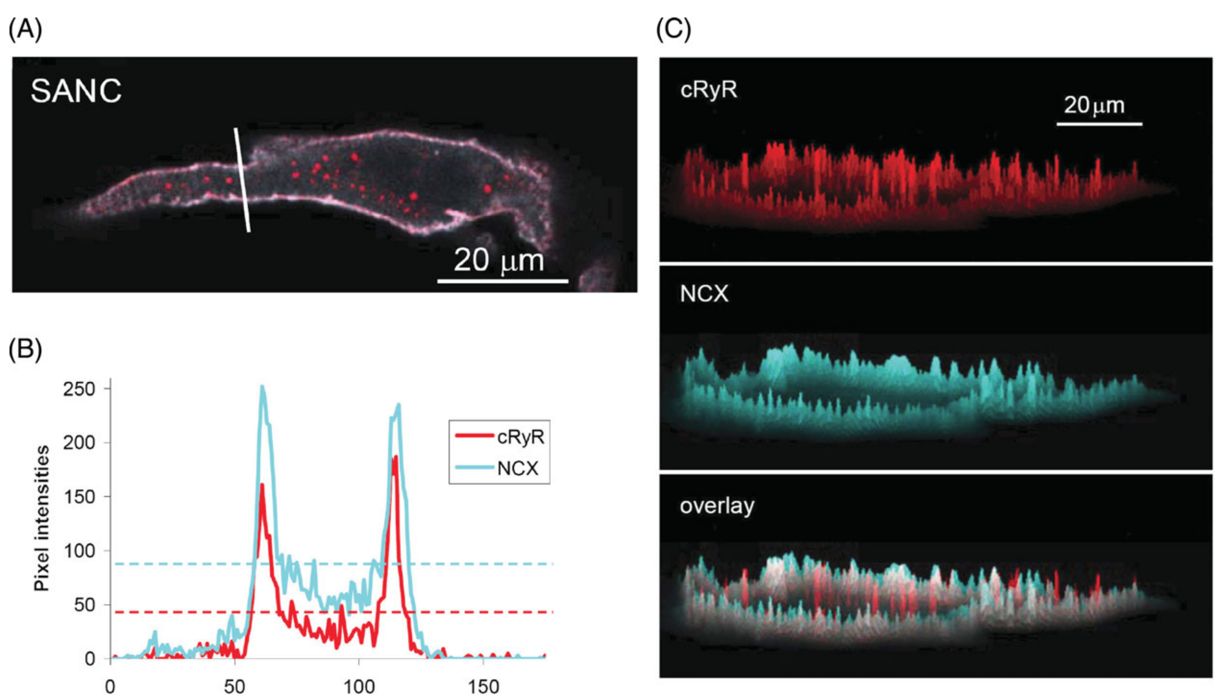

Figure 3 Morphological integration of Ca^{2+} cycling proteins with cell surface membrane proteins. (*A*) Confocal whole cell image of cells doubly immuno-labelled for Na^{+}/Ca^{2+} exchanger (NCX) and ryanodine receptor (RyR). (*B*) Graphed pixel-by-pixel fluorescence intensities of labelling along an arbitrary line, positioned as indicated by thick white lines in *A*. The horizontal dashed lines report the average pixel intensity. (*C*) Shows the topographical profiles of the pixel intensity levels of each antibody labelling and overlay of the small sinoatrial nodal cells (SANC). The maximum height represents the brightest possible pixel in the source image (using an 8-bit image intensity scale). Less bright pixels are accordingly scaled to a smaller height (from[55]).

NCX and L-type Ca^{2+} channel activation to generate rhythmic APs. Although stochastic in nature, individual LCRs become under tight control. They are entrained by regular APs to become approximately periodic and synchronized, i.e. rhythmic and powerful enough to ignite those regular APs from the voltage levels optimized by the afterpotentials.

Another aspect of the entrainment of the Ca^{2+} clock by the M clock is that Ca^{2+} influx via L-type Ca^{2+} channels also 'refuels' the Ca^{2+} clock by supplying Ca^{2+} to be pumped into the SR. The importance of this integration for sustained operation of the Ca^{2+} clock is illustrated in voltage clamp experiments:[46] when the membrane potential of intact rabbit SANC is clamped at the MDP~ -65 mV, rhythmic LCRs continue to occur, increase to a maximum, but then become damped, and cease after a few seconds (*Figure 2C*), as the cytosol and SR become Ca^{2+} depleted, due to Ca^{2+} extrusion from the cell via NCX in the absence of Ca^{2+} influx via I_{CaL}. Thus, the Ca^{2+} clock cannot sustain its long-term operation in SANC, without its partner, M clock, either; and the robust operation of the system generating rhythmic APs emerges from dynamical interactions of the two clocks. When either clock is inhibited or uncoupled from its partner, the system fails (*Figure 2B* and *C*).

7. Dynamic interaction of Ca^{2+} and membrane clocks. III: novel robust mechanism of pacemaker rate regulation

The interaction of Ca^{2+} and M clocks is, in fact, much more complex than outlined above, especially when the system is challenged by neurotransmitters to generate 'fail-safe' impulses with greatly varying rates. The Ca^{2+} cycling proteins of the SR, the NCX, L-type Ca^{2+} channels, and other sarcolemmal ion channels, mutually entrain, and thus tightly control/support each other during both basal and β-AR-stimulated spontaneous SANC firing. Multiple control points and feedback loops (i.e. interactions) are generated by the functional dependencies of the involved proteins on Ca^{2+}, cAMP, phosphorylation, and membrane potential (*Figure 4A*).

The speed at which Ca^{2+} clock ticks and generates LCRs is variable. The variability of the Ca^{2+} clock speed is linked to the rate of SR refilling with Ca^{2+}.[74,75] In turn, the variability of cycle length among different cells is directly correlated with variation in the LCR period among cells[46] and the correlation is preserved under a great variety of perturbations of SANC function, indicating that those two parameters are uniquely/fundamentally coupled (*Table 1*). A slope coefficient of unity plus a delay of 65–110 ms relates LCR period to the spontaneous cycle length in all cases. Independently of beating rates varying from about 1.2 to 4 Hz, LCRs always appear synchronously at 80–90% of the cycle length,[46,69] i.e. shortly before membrane excitation. This time window is within the so called '1:1 entrainment zone' in the phase response curves of SANC to external rhythmic electric pulses.[76] Thus, one interpretation of the unique relationship in *Table 1* is that LCRs/I_{NCX} signals can easily entrain automaticity at the greatly varying rates in rabbit SANC. Importantly, the LCR period and the cycle length remain strongly coupled, not only in the steady-state beating, but also during stringent transitions, such as the transient state after removal of voltage clamp at the MDP.[46]

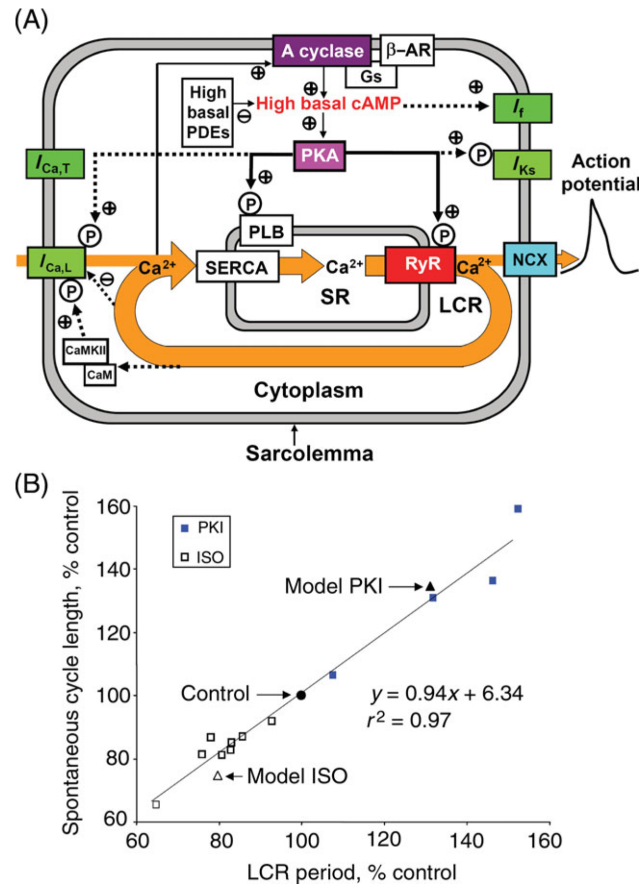

Figure 4 (*A*) Schematic illustration of functional integration and regulation of membrane and submembrane Ca^{2+} cycling to control pacemaker function via Na^+/Ca^{2+} exchanger (NCX)-mediated ignition of rhythmic action potentials and resetting of Ca^{2+} cycling by I_{CaL} -triggered CICR. The thick line indicates spontaneous sarcoplasmic reticulum (SR) Ca^{2+} cycling. Modified from[18]. (*B*) The relative PKI and isoproterenol (ISO) effects to alter cycle length over a wide range are linked to their effects on the local Ca^{2+} releases (LCR) period within the same cells. Square symbols and solid line depict the experimentally obtained data; triangular symbols depict data simulated by a numerical sinoatrial nodal cells (SANC) model using experimentally measured changes in LCR characteristics and phase. Modified from[18].

As noted in section 4, a relatively high basal phosphorylation of Ca^{2+} cycling proteins by PKA is required for basal SANC beating. Due to the unique relationship between LCR period and the cycle length (*Table 1*), the relative change in AP firing rate among cells and the relative LCR period prolongation by PKI (a PKA inhibitor), or relative LCR period reduction by β-AR stimulation are extremely highly correlated, ($r^2 = 0.97$); this relationship lies on the line of identity and embraces the entire physiological range of rhythmic AP firing of SANC (*Figure 4B*).[18,58] In response to β-AR stimulation the SR Ca^{2+} load in rabbit SANC increases by 31%.[24] When phospholamban is phosphorylated, higher SR Ca^{2+} pumping allows to reach these higher SR loads for stronger spontaneous release in shorter times. There is some evidence that phosphorylated RyRs can exhibit increased open probability[77] and more synchronized activation than in the non-phosphorylated state.[78] Although modulation of RyR function by phosphorylation remains unclear and underexplored, the above changes explain, at least in part, why LCRs become significantly larger in size

Table 1 An extremely tight link between the local Ca^{2+} release (LCR) period and the spontaneous cycle length (CL) of sinoatrial nodal cell (SANC) over a wide range of conditions ('Experimental paradigm') has been identified experimentally (from[38])

Experimental paradigm	*a*	*b*(ms)	r^2
Different SANC	CL = 0.86 × LCR period	+89	0.85
SR Ca^{2+} repletion following SR Ca^{2+} depletion	CL = 0.90 × LCR period	+85	0.86
Ryanodine	CL = 1.00 × LCR period	+110	0.90
Suppression of SR Ca pump	CL = 0.97 × LCR period	+98	0.85
PKA inhibition (PKI)	CL = 0.93 × LCR period	+90	0.92
PKA inhibition (H89)	CL = 0.97 × LCR period	+86	0.85
Basal PDE inhibition induced reduction in cycle length	CL = 0.93 × LCR period	+69	0.89
β-AR stimulation induced reduction in cycle length	CL = 1.03 × LCR period	+65	0.84

Cycle length = a × LCR period + b.

under β-AR stimulation.[24] Thus, β-AR stimulation not only shortens the LCR period but also increases LCR abundance (signal mass),[18] which is important (according to our hypothesis) to insure stronger LCR/NCX signals for AP ignitions at higher rates from lower voltages.

Although interactions of Ca^{2+} and M clocks are rather complex and remain underexplored, modulatory changes of ion channel characteristics produced by Ca^{2+}, calmodulin, CaMKII, cAMP, and PKA can be viewed as supporting the enhanced Ca^{2+} clock operation (dotted lines in *Figure 4A*), as they tune and optimize ignition of APs by LCRs; for example: (i) a larger Ca^{2+} influx via a larger and more frequent Ca^{2+} current enhances the rate of SR Ca^{2+} pumping and reloading; (ii) a shift in I_f activation limits hyperpolarization, and thus optimizes the voltage for I_{CaL} ignition by LCR/I_{NCX}.

Thus, the speed at which Ca^{2+} clock ticks and generates LCRs is variable, matching the chronotropic demand for a given condition, and is governed by the SR Ca^{2+} loading and Ca^{2+} release characteristics, which in turn, are governed by the degree of phosphorylation of its aforementioned Ca^{2+} cycling proteins. This novel rate regulation mechanism is not described by classical theoretical SANC models (section 1) because they do not account for the diastolic Ca^{2+} release. One model[79] explains the effect of β-AR stimulation on the basis of a prior experimental observation that β-AR stimulation shifts I_f activation curve towards more positive voltages.[80] Since I_f contributes to the early-mid DD, this model predicts a larger slope of the early DD resulting in earlier attaining an excitation threshold (*Figure 5A*). While, the I_f contribution to β-AR stimulation effect cannot be measured directly, a recent experimental study[81] provided evidence for this membrane-delimited mechanism by analysing DD shapes in rabbit SANC. However, that study failed to analyse the contribution of the late non-linear DD component. When the entire DD is analysed it becomes clear that while both early linear and late non-linear components of DD undergo substantial changes in SANC under β-AR stimulation, it is the non-linear component, linked to diastolic LCR activation, that contribute mainly to the rate acceleration of cell beating.[68] A minor role of I_f in mediating the β-AR acceleration of SANC firing rates, in fact, has been long known (since 1983)[11] because adrenaline produces a marked positive chronotropic effect in SAN under the conditions when I_f is blocked by Cs^+.[11] This small Cs^+ effect was later confirmed in isolated SANC stimulated by isoproterenol.[24] Finally, evidence that I_f is not required for heart rate acceleration has been recently reported in mice with deleted HCN4 (Herrmann *et al.*, EMBO J; 2007:26,4423–32, published while this review was in print).

In contrast to the classical SANC model described above, a numerical model that includes LCRs predicts more closely experimental data (including the fine structure of the entire DD).[68] As more abundant LCRs appear earlier in the cycle, a stronger diastolic NCX current also appear early, thus resulting in an earlier acceleration of DD and a shorter cycle length (*Figure 4B* 'model ISO' and *Figure 5B*). A similar mechanism of rate regulation mechanism by β-AR stimulation is also predicted by our most recent model which features spontaneous Ca^{2+} release that is controlled by SR Ca^{2+} load[75] (data not shown).

The role of RyR-initiated LCRs in mediating the chronotropic response to cAMP-dependent signalling by β-AR stimulation has been tested in experiments, in which ryanodine was applied to interfere with Ca^{2+} release. In the intact organism, *in vivo*, [18] the increase of heart rate elicited by β-AR stimulation, using microdialysis of isoproterenol via the SAN artery, is markedly blunted in the presence of ryanodine. *In vitro*, in single SANC, a markedly blunted effect of β-AR stimulation in the presence of ryanodine has reported in two studies,[24,82] and this effect occurs even with β-AR mediated increase in I_{CaL} remaining intact.[24] However, based upon an observation that effects of a membrane-permeable cAMP (CPT-cAMP) in control and ryanodine-treated SANC are similar (~17.7 vs. 17.3% rate increase), it has speculated that cAMP-mediated I_f activation is somehow perturbed by a 'Ca^{2+}-dependent interference' in ryanodine-treated cells.[82] A more recent study clearly shows: CPT-cAMP produces smaller effect (12.5%) in ryanodine-treated SANC, and a much larger effect (36.3%) in control cells (see Supplementary material online, *Figure S3* in[18] for details). Thus, these data indicate that the increase in SANC firing rate either by a β-AR stimulation or exogenously applied cAMP, is mediated mainly via the ryanodine-sensitive, cAMP/PKA-modulated Ca^{2+} release mechanism, rather than Cs-sensitive, cAMP-modulated I_f. Since I_f is activated by cAMP but not by PKA, the fact that PKA inhibition markedly reduces SANC AP firing rate, provides additional evidence for a crucial PKA-dependent component in the SANC response to β-AR stimulation. After a prolonged time of submaximal PKA inhibition, or following higher levels of PKA inhibition, LCRs are no longer detectable, Ca^{2+} and M clocks become uncoupled, and the rate of spontaneous APs markedly slows, becomes highly irregular and often ceases.[18,83]

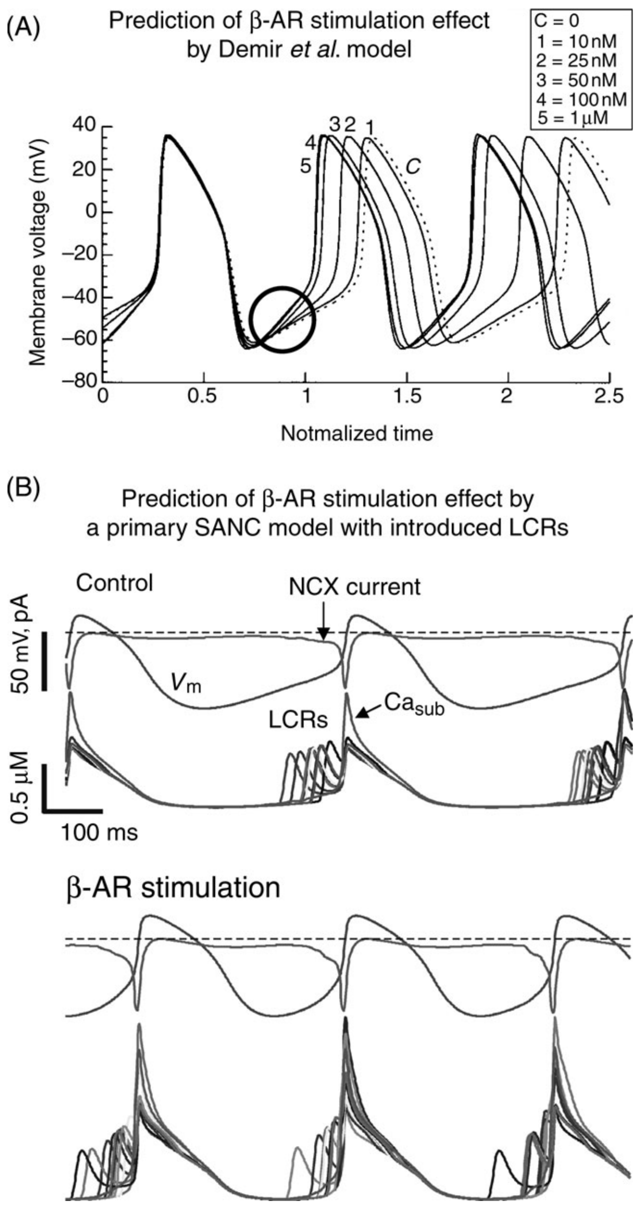

Figure 5 An essentially membrane-delimited sinoatrial nodal cells (SANC) model and a model that includes local Ca^{2+} releases (LCRs) predict different mechanisms of pacemaker rate regulation by β-adrenergic receptors (β-ARs). (*A*) In a classical model isoprenaline regulates the cycle length by varying the rate of early diastolic depolarization (DD) (circle) mainly via I_f modulation. Labels at the second action potential (AP) indicate effects of various isoprenaline concentrations (from[79], used with permission). (*B*) In a SANC model, featuring stochastic LCRs, β-AR stimulation accelerates both early and late DDs. As larger-size LCRs appear earlier in the cycle, a stronger diastolic Na^+/Ca^{2+} exchanger (NCX) current also appears at an earlier time following the previous AP, thus resulting in an earlier acceleration of late DD and a shorter cycle length. Membrane potential (V_m), NCX current, overlapped arbitrary eight LCRs, and average submembrane $[Ca^{2+}]$ (Ca_{sub}) are depicted. Modified from[18].

8. Robustness and complexity of the sinoatrial nodal cells pacemaker system

The cardiac pacemaker is extremely robust: over the lifespan it generates billions of fail-safe, uninterrupted heartbeats of greatly varying rates. Robustness, i.e. ability to maintain stable functioning despite perturbations, is a perceived fundamental property of complex systems including biological systems, characterized by extreme heterogeneity, multiple feedback loops, and redundancies.[84,85] For example an extremely robust system of two coupled oscillators, referred to as a segmentation clock, directs development of the embryo.[86] A simple mechanism featuring a mere basic functionality under ideal conditions cannot be robust. The concept of robustness can be illustrated by a comparison of a small sport airplane (simple) with a large transcontinental airliner (complex, equipped with automatic flight control system, *Figure 2* in[85]): both can fly, but only the latter safely flies long distance in various weather conditions. While the cardiac pacemaker has been, and continues to be considered essentially on the basis of a rather simple operation of ion channels,[3] our hypothesis suggests and experimental data document that it is, in fact, a robust complex system with multiple feedback mechanisms and redundancies (*Figure 4A*). Redundancies include several regulation pathways of same molecules (e.g. L-type Ca^{2+} channels regulated by Ca^{2+}, PKA, and CaMKII) and a heterogeneous redundancy of oscillators: two coupled oscillators of different nature operate in SANC, i.e. electrical M clock and chemical Ca^{2+} clock. Interestingly, a tight integration of intracellular Ca^{2+} signals and cell membrane electrogenic processes has been recently discovered in other types of vital pacemakers, including those controlling circadian, respiratory, and intestinal rhythms,[87–90] indicating that a higher level of complexity is required for emergence of robust, 'fail-safe' pacemaker cell function, regardless of the particular pacemaker type.

9. Summary

Based on evidence presented here we suggest a new theory of cardiac pacemaker function: a complex system driven by coupled oscillators, i.e. Ca^{2+} and M clocks, and regulated by multiple feed-back and feed-forward regulatory mechanisms. It is important to note, that our view on cardiac rhythm initiation (formulated also in a recent numerical model)[75] does not deny or contradict conventional ideas and formulations of the M clock, but includes them entirely as a counterpart of the Ca^{2+} clock within the robust SANC system. This gives a new, relatively simple interpretation of the entire DD shape, a major problem of the cardiac pacemaker field since its discovery by Arvanitaki *et al*.[91] working on the snail heart in 1937. Ca^{2+} clock rhythmically ignites subsequent AP via DD acceleration, thus initiating new pacemaker cycle. Accordingly, events that underlie the early DD can be interpreted as an afterpotential, i.e. the process of membrane recovery following the prior AP.

Since all cardiac cells have both membrane ion channels that generate APs, and SR Ca^{2+} cycling, the concept of the interacting clocks presented here for pacemaker cells can be extended to ventricular cells in which mutual entrainment of membrane and Ca^{2+} clocks drive rhythmic contractions. Thus a general theory of the rate and strength of the heartbeat emerges from the concept of coupled Ca^{2+} and membrane potential oscillators. This general theory may provide a new key to understanding the plethora of unsolved mysteries of normal and pathological heart function.

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