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A Paradigm Shift for the Heart's Pacemaker

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Introduction

About 50 years ago, quantitative Hodgkin-Huxley formalism of membrane excitation was assimilated into classical cardiac pacemaker cell dogma as its main tenet: Voltage- and time-dependent gating properties of an ensemble of mutually interacting surface membrane ion channels (Fig. 1 blue) governs the rate and rhythm of spontaneous action potential (AP) firing of the heart's pacemaker cells by controlling the spontaneous membrane depolarization between APs. Because this ensemble of electrogenic membrane proteins can, in silico, generate rhythmic APs, it can be envisioned as a surface membrane "clock" (M clock) (c.f. for review¹). In the context of the APs that it produces, the M clock regulates cell Ca^{2+} balance in a given steady state via Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} efflux through the Na^+ - Ca^{2+} exchanger (NCX) (Fig. 1). (In order to assist comprehension of the complex system involving coupling between surface membrane molecules and intracellular Ca^{2+} cycling molecules, it is suggested that the reader refer to the figures as these are discussed in the text.)

The electrogenic and regulatory molecules on the surface membrane of sinoatrial nodal cells (SANC) are strongly modulated by Ca^{2+} and phosphorylation (Fig. 1, red arrows). More recently² it has been discovered that phosphorylation-dependent intracellular Ca^{2+} cycling (Fig. 1, within gray area) is also an integral player in controlling pacemaker cell automaticity. Spontaneous, localized and critically timed Local subsarcolemmal Ca^{2+} Releases (LCR's) generated by sarcoplasmic reticulum (SR) via ryanodine receptors (RyR) (Fig. 1) occur during the late diastolic depolarization (DD). The SR has been referred to as an intracellular " Ca^{2+} clock" because the submembrane Ca^{2+} oscillations that it generates are periodic, occur when surface membrane function is experimentally eliminated³, and also exist in silico⁴. But, in nature, neither the M clock, nor the Ca^{2+} clock, exists in isolation of each other: Ca^{2+} and phosphorylation-dependent coupling of the functions of molecules comprising the subsystem M and Ca^{2+} clocks creates a complex, robust, coupled PACEMAKER CLOCK SYSTEM (Fig. 1) that ensures stable rhythmic firing of cardiac pacemaker cells. Autonomic receptor stimulation confers flexibility to the rate at which the pacemaker ticks^{2,4–7}.

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The coupled-clock pacemaker system

During each SANC duty cycle, the M clock and Ca^{2+} clock are continuously coupled and affect Ca^{2+} release at two specific times: during the late DD and following the AP upstroke. The coupled-clock system keeps the time that has elapsed from prior AP and generates critically timed LCRs during the late DD to prompt the occurrence of the subsequent AP^{2,3,5-7}. Confocal imaging of Ca^{2+} in SANC, combined with non-invasive perforated patch-clamp electrophysiology, has documented that RyR-generated, sub-sarcolemmal LCR's emerge after a delay following the upstroke of the global cytosolic transient effected by the prior AP (Fig. 2A), and grow in magnitude during the DD, peaking during the late DD, as they merge into the global cytosolic Ca^{2+} transient triggered by the next AP^{3,5,8,9}. Voltage clamp studies of SANC show that rhythmic LCRs generate miniature, rhythmic current fluctuations due to NCX current (I_{NCX}) activation^{3,5,8}. While regulated by membrane potential and submembrane Ca^{2+} , NCX does not have time-dependent gating, as do ion channels, but generates an inward I_{NCX} almost instantaneously when submembrane $[\text{Ca}^{2+}]$ increases. Analysis of the fine DD structure indicates that LCR occurrence and summation of LCR-activated NCX currents cause an exponential increase in late DD⁵. This LCR-induced, I_{NCX} -executed surface membrane "prompt" facilitates achievement of a membrane potential to activate a sufficient number of voltage-gated L-type Ca^{2+} channels, leading to generation of the rapid AP upstroke. This surface membrane "prompt" is crucial for generation of the AP, the "main event" of pacemaker cell function, because experimental evidence documents that inhibition of I_{NCX} by acute exposure to Na^+ -free bathing fluid during DD immediately abolishes spontaneous AP firing^{8,10}.

Regulation of the coupled-clock basal "ticking speed"

The delay between the onset of the global cytosolic transient triggered by the prior AP and the emergence of an LCR during the subsequent DD is the LCR period, which is a "readout" of the kinetics of coupled pacemaker clock restitution process that the "ticking speed" of the coupled-clock system (Fig. 2A lower)¹. L-type Ca^{2+} channels, NCX and SR Ca^{2+} cycling are critical nodes within the coupled-clock system because their interactions are the essence of the system's ability to function as a clock, i.e. to generate rhythmic LCRs of a stable period that prompt the generation of spontaneous APs at a stable rate. During an AP, the global SR Ca^{2+} release via Ca^{2+} -induced Ca^{2+} release (CICR) depletes the SR network of Ca^{2+} . This SR Ca^{2+} depletion turns off the spontaneous LCRs. The clock restitution occurs between the time of global SR Ca^{2+} depletion by CICR of the prior AP and achievement of a threshold intra-SR $[\text{Ca}^{2+}]$ and removal of RyR inactivation that are required for subsequent spontaneous RyR activation that generates LCRs during late DD. The Ca^{2+} extruded from the cell via NCX during each cycle is replenished via Ca^{2+} influx via L-type Ca^{2+} channels that is pumped into the SR (Fig. 1). Thus, continuous Ca^{2+} pumping continues to refill the SR with Ca^{2+} following Ca^{2+} depletion by the AP. In other words, during the restitution period, i.e. the LCR period, the Ca^{2+} clock is always ticking and keeping time. This Ca^{2+} replenishment is a critical component of the process to generate LCRs guarantees the achievement of a threshold level of intra-SR Ca^{2+} , the oscillatory substrate, required for the SR to resume generation of spontaneous LCRs. NCX-mediated clearance of Ca^{2+} released into the submembrane space is also crucial for a given steady state level of LCR activation of NCX during the subsequent DD because RyR activation depends on both intra- and extra- SR $[\text{Ca}^{2+}]$. Specifically, while intra-SR level gradually increases after the AP due to SR Ca^{2+} pumping, the extra-SR $[\text{Ca}^{2+}]$ level (i.e. submembrane $[\text{Ca}^{2+}]$) decreases partly due to the NCX-mediated Ca^{2+} extrusion, so that LCRs begin to occur only when these two process meet a requirement of a sufficient probability for RyR to open and to generate LCRs⁴. A given steady state has unique, stable restitution kinetics that guarantees stable beat-to-beat LCR periodicity within that steady state.

PKA- and CaMKII-dependent phosphorylation of coupled-clock proteins (Fig. 1, red) are also critical determinants of the coupled-clock function. Phosphorylation of SR Ca^{2+} proteins, e.g., SERCA2 and RyR (Fig. 1 gray area), regulates the kinetics at which SR cycles Ca^{2+} , the clock's oscillatory substrate; and phosphorylation of surface membrane electrogenic proteins, e.g., I_{CaL} , and I_{K} (Fig. 1 blue) regulate the cell Ca^{2+} balance during each cycle. Regulation of SR Ca^{2+} cycling and membrane ion current generators by a common set of factors (Fig. 1 red labels) couples SR and surface membrane protein functions⁴, to produce a robust, complex biological pacemaker system with numerous functional redundancies (Fig. 1). Ca^{2+} within the submembrane space binds to calmodulin to activate adenylyl cyclase (AC). Even in the absence of β adrenergic receptor (β -AR) stimulation, Ca^{2+} -activated constitutive AC activity in SANC¹¹ generates a high basal level of cAMP. Note, that while cAMP is required for the activation of (Fig. 1 red) PKA and for PKA-dependent protein phosphorylation, cAMP does not directly couple to SR Ca^{2+} cycling proteins (Fig. 1). It does, however, directly bind to HCN channel sub-units (vide infra). SANC also have a high level of basal CaMKII activation. The coupled system pacemaker clock remains stable in a given steady state because this "feed-forward" Ca^{2+} signaling, i.e., Ca^{2+} release begets Ca^{2+} release (Fig. 1 gray shading), is kept in check by factors that regulate Ca^{2+} , or AC, PKA or CaMKII activity. High basal phosphodiesterase (PDE) activity within SANC is one such control mechanism (Fig. 1)⁶. Basal phosphoprotein phosphatase activity, while not yet characterized in SANC, may be another control point (Fig. 1). In a given steady state, the average LCR period of each AP cycle is stable or "failsafe" because the levels of phosphorylation of M and Ca^{2+} clock proteins is stable, and the steady state balance of cell Ca^{2+} that is upset by events that occur during an AP, completely restitutes by the onset of the late DD, when LCR occurrence resumes. Thus, the AP that is "prompted" via surface membrane depolarization due to LCR-activated I_{NCX} during the late DD, guarantees its own rhythmic occurrence in future cycles by its effects on the Ca^{2+} clock: the AP (i) stops LCRs, via SR Ca^{2+} depletion and RyR inactivation that reset the LCR period and (ii) rewinds the Ca^{2+} clock by replenishing its Ca^{2+} load via Ca^{2+} influx. This guarantees the timely occurrence of LCRs during late DD that provides timely membrane depolarization "prompts" in subsequent cycles, thus insuring the continued occurrence of "on-time", rhythmic APs in a given steady state rate. This critical mutual dependence of the M and Ca^{2+} clocks, leads to their mutual entrainment, which renders the coupled pacemaker clock system robust, i.e. stable function in a given steady-state. This concept of mutually entrained molecular function as the basis of the coupled-clock pacemaker function of SANC is supported by novel numerical simulations⁴ (Fig. 2D).

In addition to maintaining a stable LCR period (i.e., a stable coupled clock "ticking speed") in the basal state, the heart's pacemaker clock must tick over a wide range of LCR periods to generate APs at rates that encompass the complete physiologic range. Variation in steady-state coupled clock system ticking speed is achieved by gradations in coupling factors that modulate the system component proteins (Fig. 1 red) that lead to gradations of the steady state cell Ca^{2+} balance and to gradations in the kinetics of SR Ca^{2+} cycling. In other words, different steady-state conditions produce gradations in the LCR period that lead to changes in the timing of LCR activation of I_{NCX} , resulting in gradations in the timing of the onset of the exponential phase of late DD membrane depolarization, and therefore to gradations of AP cycle lengths (Fig. 2D).

Autonomic receptor stimulation: Nature's way to ensure stable AP generation at different steady-state rates

Nature imparts flexibility to the pacemaker clock's "throttle" via variation in G protein coupled receptor (GPCR) signaling (Fig. 1, green) that links these receptors to the very same factors (Fig. 1 red) that regulate basal state LCR period. Essentially, GPCR signaling via effecting variation in the degree of PKA- and CaMKII-dependent protein phosphorylation within the

coupled-clock system, causes in variation in steady state transsarcolemmal ion fluxes and SR Ca^{2+} cycling kinetics that shift the coupled-clock LCR period, leading to shifts in the spontaneous AP firing rate (Fig. 2D).

β -adrenergic receptor stimulation

β -AR stimulation (or PDE inhibition) specifically increases the integrated LCR signal mass (LCR number, amplitude and width) and recruits additional RyRs to generate spontaneous LCRs^{2,6,9}. During β -AR stimulation, in conjunction with an increase in PKA-dependent phosphorylation, the LCR period becomes markedly shorter (Fig. 2B), shifting LCR occurrence to an earlier time during DD, i.e., a time occupied solely by early DD mechanisms during basal state beating^{5,12}. This effect often obliterates a linear early DD. As a result of the reduction in the LCR period by β -AR stimulation (or PDE inhibition), the cycle length becomes concomitantly reduced (Fig. 2C).

Normal acceleration of the beating rate of isolated SANC^{9,13,14} cannot be achieved without normal SR Ca^{2+} cycling, even when the I_{CaL} augmentation and I_{f} activation by β -AR signaling remains intact. This is another indicator of the mutual interdependence of the M and Ca^{2+} clocks. In this scenario, SERCA2 Ca^{2+} pumping and RyR Ca^{2+} release fluxact as a “switchboard” system or node that links β -AR stimulation to an increase in SANC firing rate. (The specific switchboard function is to generate LCRs of different steady state periods, dictated by changes in phosphorylation of SR proteins that directly regulate Ca^{2+} cycling kinetics or surface membrane proteins that regulate cell Ca^{2+} balance.) It has also been documented that acceleration of the sinus rhythm by β -AR stimulation in the primary region of isolated canine SAN¹⁵, or in the intact dog heart² requires intact intracellular Ca^{2+} dynamics. Recent studies demonstrate that the positive chronotropic effect of β -AR stimulation in mice also requires CaMKII-dependent activation¹⁶.

While cAMP binding to HCN4 modulates I_{f} activation (Fig. 1), studies in which either HCN2 or HCN4 channel were genetically modified in mice, and prior studies in which I_{f} current was blocked in numerous species by ionic or pharmacological maneuvers, do not indicate that I_{f} is required for the SANC basal beating rate, or for its modulation by β -AR stimulation. (cf^{12,13} for detailed recent reviews). In contrast, key roles of I_{f} may be to prevent clamping of the SA node primary pacemaker area by a relatively low membrane potential of surrounding SAN atrial tissues, and to stabilize the AP rhythm during rhythm transitions (⁴ and also cf¹³ for review).

Cholinergic receptor (CR) stimulation

Activation of vagal tone suppresses the SA node spontaneous beating rate through several G_i protein coupled downstream targets, including $\text{Gi}\beta\gamma$ activated I_{KACH} , accompanied by membrane hyperpolarization and $\text{Gi}\alpha$ and $\text{Gi}\beta\gamma$ inhibition of AC activity (fig. 1 green). It was formerly thought that CR stimulation, like that β -AR stimulation, induces chronotropic effects entirely by an effect on sarcolemmal membrane ion channels, specifically I_{KACH} , I_{f} , and I_{CaL} . Recent studies, however, demonstrate that CR stimulation-induced reduction of AC activity and cAMP levels^{7,17}, leads to a reduction in PKA-dependent phospholamban phosphorylation¹⁴ and that LCR's¹⁴ or their counterpart, late diastolic Ca^{2+} elevation¹⁷ are also suppressed. For example, at IC_{50} , the CR ligand, carbachol (CCh), reduces cAMP, and reduces PKA-dependent phospholamban phosphorylation by ~50% (Fig. 2B), causes a reduction in LCR number and size, and an increase in the LCR period (Fig. 2B) that parallels the coincident AP cycle prolongation (Fig. 2C). A similar effect results from inhibition of PKA activity by a PKI, a specific PKA inhibitory peptide². Phosphatase inhibition reverses the effect of CCh on SANC LCRs and AP cycle length⁷. Thus, at low levels (i.e. physiological) of CR stimulation, when I_{KACH} activation is not evident⁷, AP cycle length prolongation is

attributable to a suppression of cAMP-mediated, PKA-dependent Ca^{2+} signaling of the coupled M and Ca^{2+} clock system. As the intensity of CR stimulation increases (beyond IC_{50} for CCh), a tight coupling between suppression of PKA-dependent Ca^{2+} signaling of the coupled clock and I_{KACH} activation within the M clock underlies a more pronounced reduction in the beating rate⁷.

In summary, GPCR stimulation, or interventions that affect PKA-dependent signaling distal to the GPCRs, leads to phosphorylation-dependent shifts in the LCR period (Fig. 2B) and in AP cycle length that occur simultaneously with shifts in the LCR period over the full physiologic range of cycle lengths (Fig. 2C). In other words, GPCR stimulation amplifies or dampens the very same mechanisms that control the basal state LCR period and AP cycle length. The phosphorylation of phospholamban at serine 16 provides an easily measured, informative index of the status of PKA-dependent protein phosphorylation of the coupled-clock system in a given steady state^{2,6,9}. Fig. 2B illustrates the relationship between the steady-state levels of phospholamban phosphorylation and the in the steady state LCR period of the coupled clock. The strong coupling of the LCR period and cycle length (Fig. 2C) is predicted by simulations of a novel numerical model of rabbit SANC⁴ (Fig. 2D, “various predicted LCR periods” vs. cycle length decrease/increase). Note bene, in Fig. 2B that changes in phosphorylation status of numerous other M and Ca^{2+} proteins in addition to phospholamban phosphorylation occur and are undoubtedly important to the system clock response to GPCR stimulation^{2,4-7,9,13} but have not yet been directly measured. Changes in amplitude in I_{CaL} and I_{K} in response to β -AR stimulation (or PDE inhibition) surely reflect changes in phosphorylation state of these channel proteins.

A modern VIEWPOINT on cardiac pacemaker cell automaticity

On the basis of recent experimental evidence^{1-3,5-11,13-17}, which is supported by novel numerical modeling⁴, it is reasonable to conclude that SANC normal automaticity is controlled by a complex SYSTEM of clocks comprised of tightly coupled SR Ca^{2+} cycling molecules and surface membrane molecules (Fig. 1) that become functionally, mutually entrained. The same factors that regulate SR Ca^{2+} cycling, i.e., Ca^{2+} and PKA and CaMKII dependent protein phosphorylation (Fig. 2), not only interact with each other, but also modulate sarcolemmal ion channel function. Thus, these coupling factors are the critical nodes within the coupled clock pacemaker system that ensure the robustness and stability of the coupled pacemaker clock system because entrain the functions of SR Ca^{2+} cycling and surface membrane proteins. GPCR signaling ensures pacemaker firing rate flexibility by effecting rate regulation via modulation of these very same Ca^{2+} - and phosphorylation-dependent factors that ensure robust, “fail safe”, pacemaker operation in the basal state. The intimately intertwined robustness and flexibility of the heart’s pacemaker system ensures a wide physiologic range of stable heart rates that is required for peaceful rest, or flight or fight.

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The LCR period, its regulation by PKA-dependent phosphorylation, and its impact on the SANC spontaneous cycle length.

Fig. 2.

A. The LCR period of the coupled pacemaker clock system. Top--Simultaneously measured confocal line scan image of sub-sarcolemmal Ca^{2+} and membrane potential in a representative rabbit SANC. Arrows indicate LCR occurrence during DD. Bottom-- Definition of LCR period and AP cycle length (Modified from ⁵). B. The relative effects of a specific peptide PKA inhibitor (PKI), carbachol (CCh), phosphodiesterase (PDE) inhibition by milrinone or isobutylmethylxanthine (IBMX), and isoproterenol (ISO) to alter the LCR period are linked to their effects on the phospholamban (PLB) phosphorylation. The dashed line is the best fit least squares logarithmic function through the points: $Y = -52.01 \ln(X) + 107.11$ ($R^2 = 0.92$). C. The relative effects of PKI, CCh, PDE inhibition and ISO to alter the spontaneous cycle length over a wide range are linked to their effects on the LCR period. The least squares linear function through the points is: $\text{cycle length} = 0.89 \text{ LCR period} + 11.43 \text{ ms}$, $r^2 = 0.97$. The dashed line is the line of identity. D. A novel numerical SANC model of a dynamically integrated system of Ca^{2+} and membrane clocks predicts the wide range of pacemaker rate modulation via variations in SR Ca^{2+} pumping rate (color coded, 1 to 30 mM/s), mimicking various degrees of PKA-dependent phospholamban phosphorylation. Shown are simultaneous simulations of SR $[\text{Ca}^{2+}]$, SR Ca^{2+} release flux, I_{NCX} , and V_m . Modified from ⁴.