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The funny current in the context of the coupled clock pacemaker cell system

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A coupled-clock system, not just a “Ca²⁺-clock” or an M-clock, is the essence of pacemaker cell function

It has been now widely documented in many laboratories in many species (review¹) that Local Ca²⁺ releases (LCRs), generated by the sarcoplasmic reticulum (SR) via spontaneous ryanodine receptor (RyR) activation, emerge during early DD and, grow in magnitude during the DD, and peak during late DD, forming an LCR ensemble Ca²⁺ signal, i.e. late diastolic Ca²⁺ elevation (LDCaE)(Fig.1A-C). LCRs nearly instantaneously generate inward Na⁺/Ca²⁺exchange (NCX) current fluctuations that depolarize the surface membrane, prompting the rapid action potential (AP) upstroke (Fig.1D). SR has been referred to as an intracellular “Ca²⁺-clock” because LCRs are roughly periodic¹. The delay between the AP-triggered global cytosolic Ca²⁺transient and LCR emergence during DD is the LCR period (Fig.1A). Variations in the SANC AP cycle length are predicted by variations in the LCR period (vide-infra).

But the concept that Ca²⁺-clock properties, per se, regulates the SANC LCR period and automaticity is **strictly** incorrect. The LCR period is regulated not only by the kinetics of SR Ca²⁺ pumping and RyR characteristics but also by the quantity of intracellular Ca²⁺ made available for pumping into the SR via sarcolemmal Ca²⁺ flux (Fig.2A) regulated by membrane ion channels. The contributions of L-type Ca²⁺ channels (LCCh) and NCX to the Ca²⁺ balance and, hence, LCR period are obvious. But K⁺ channels, and **even funny channels** (Fig.1D), are also indirectly implicated in the Ca²⁺balance: K⁺channels repolarize membrane potential to inactivate LCCh and to activate I_{NCX}, and funny channels limit hyperpolarization and, hence, Ca²⁺ efflux via NCX. Thus, the LCR period embraces complex interactions of cell membrane electrogenic molecules and intracellular Ca²⁺ cycling molecules (Fig.2A) and is a “readout” of the restitution kinetics that define the “ticking speed” of the coupled-clock system.

The robustness of such a coupled system and essential clocks interactions can be construed as a union and harmony of opposites, i.e. by a yin-yang analogy (Fig.1D), reflecting a dual nature of an ion: a particle/atom, i.e. subject to diffusion, pumping and release by the SR within the Ca²⁺-clock; and an electric charge functioning within the M-clock. The yin is activated during diastole as LCRs emerge at the completion of the coupled clock restitution process (the LCR period), with the achievement of a threshold intra-SR [Ca²⁺] due to

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SERCA Ca^{2+} pumping and removal of RyR inactivation, which both are required for subsequent spontaneous RyR activation that generates LCRs. The waxing of yin, i.e. the growth of submembrane LCRs, activates yang, i.e. the electrical component of the coupled system, manifest activation of I_{NCX} , which drives DD to reach a threshold of LCCh activation to generate rapid upstroke of the next AP (Fig.1D).

Although spontaneous Ca^{2+} release of yin is intrinsically stochastic, its potentially deleterious unlimited regenerative nature is quenched by yang's induction of an AP, thus shifting yin properties from stochasticity to periodicity. The yin periodicity is entrained by rhythmically occurring APs of yang via rhythmically triggered depletion of the SR. Hence, during spontaneous AP firing, the SR does not oscillate Ca^{2+} independently, as it can in the absence of M-clock¹, but rather operates as a "stop-watch" to generate a timely crucial LCR/ I_{NCX} signal during DD, and then is reset by the AP. Furthermore, while yang initially depresses yin, it simultaneously initiates the next yin cycle, as Ca^{2+} influx via LCCh restores Ca^{2+} lost from the cell via NCX earlier in the cycle. In a given steady-state, the electro-chemical, yin-yang interactions (described above) robustly recur during each cycle, i.e. each yin (or yang), initiating yang (or yin), guarantees its own future periodic existence.

Signaling within the coupled-clock system

Briefly, LCCh, NCX and SR Ca^{2+} cycling, cAMP, cAMP-mediated, PKA-dependent and Ca^{2+} -calmodulin (CaM)-dependent protein phosphorylation and Ca^{2+} itself are critical nodes within the coupled-clock system (Fig.2). The interactions they enable 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 AP's at a stable rate. In addition to its well-known modulating effects on LCCh and K^+ channels, SERCA and RyR (Fig.2), Ca^{2+} binds to CaM to activate adenylyl cyclase (AC). Phosphorylation of SR Ca^{2+} proteins, e.g., SERCA2 and RyR (Fig.2A, 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. LCCh and K^+ channels (Fig.2A, blue), regulates the cell Ca^{2+} balance during each cycle. Numerous functional redundancies (Fig.2A) and dual regulation of SR Ca^{2+} cycling and membrane ion current generators by a common set of factors (coupling factors or nodes, Fig.2A, red labels) ensures a robustness of the coupled clock.

Constitutive Ca^{2+} -activated AC activity in SANC generates a high basal level of cAMP. Thus, the coupled pacemaker system dynamics are driven by a "feed-forward" Ca^{2+} signaling, i.e., Ca^{2+} release begets Ca^{2+} release via PKA and CaMKII pathways (Fig.2A): $\text{Ca}^{2+} \rightarrow \text{Ca}^{2+}$ -activated-ACs $\rightarrow$ cAMP $\rightarrow$ PKA $\rightarrow$ phospholamban (PLB) & RyR & LCCh phosphorylation $\rightarrow$ more Ca^{2+} $\rightarrow$ CaM $\rightarrow$ CaMKII $\rightarrow$ SERCA & PLB & RyR & LCCh phosphorylation $\rightarrow$ more Ca^{2+} $\rightarrow$ etc. High basal phosphodiesterase (PDE) activity and phosphoprotein phosphatase activity confer stability to the potentially regenerative signaling of the system by modulating factors that regulate Ca^{2+} , or AC, PKA or CaMKII activity (Fig.2A). CaM-mediated LCCh inactivation is another restraining mechanism. This system of a high rate cAMP production by Ca^{2+} -activated ACs coupled to a high rate PDE degradation of cAMP yet still maintaining high basal levels of cAMP and, hence, basal phosphorylation of the signaling proteins of both clocks, is reminiscent of similar signaling systems that occur with the retina and olfactory apparatus. Such a system guarantees rapid responses in [cAMP]-PKA signaling when local $[\text{Ca}^{2+}]$ increases to increase AC activity and cAMP. The net result is that the basal LCR period and AP cycle length are maintained near the midpoint of their functional ranges (Fig.2B,C). Thus, in a given steady state, the average LCR period of each cycle is stable or "failsafe" because the levels of phosphorylation of M and Ca^{2+} -clock proteins are stable, and the cell Ca^{2+} balance that is

transiently perturbed by events that occur during an AP, completely restitutes by the onset of the late DD, when LCR occurrence resumes.

Nature imparts flexibility to the coupled clock via variation in G protein coupled receptor (GPCR) signaling (Fig.2A, green) that links these receptors to the very same nodes (Fig.2A red) that regulate basal state LCR period¹.

Specific experimental evidence for the symmetry of coupling of clocks' function within the pacemaker cell system

- **Requirement for instantaneous NCX function:** Abrupt disabling of NCX function in SANC by “spritzing” Na⁺ free medium during DD acutely retards the evolution of a normal DD and prevents the subsequent AP (review¹). Parenthetically, atrial specific NCX-knockout mice do not exhibit any atrial-initiated AP activity², despite no difference in I_f between wild-type and knock-out.
- **Requirement for cycle-to-cycle AP-generated Ca²⁺influx:** When SANC are voltage-clamped at the maximum diastolic potential (MDP), the Ca²⁺-clocks, after several oscillations, cease to function (Fig.3A-C), because the SR becomes Ca²⁺-depleted. Following removal of the voltage clamp, as I_{CaL} progressively replenishes the SR Ca²⁺, the spontaneous beating rate gradually returns to pre-clamped level, with cycle-to-cycle reductions in the LCR period predicting cycle-to-cycle reductions of spontaneous cycle length(Fig.3D).
- **Intracellular Ca²⁺ buffering transitions:** Concomitant time-dependent changes in intracellular [Ca²⁺], DD, and LCR period following introduction of a caged Ca²⁺ buffer into spontaneously beating SANC predict time-dependent changes in AP firing rate and rhythm³. A recent study, in which intracellular Ca²⁺ was not even measured⁴, failed to detect any acute reduction in AP firing rate when BAPTA was introduced into SANC via acute rupture of a membrane patch. This result can be explained by a small, artifactual rupture-induced patch seal leak current that shifts the current balance of tiny DD currents offsetting the true BAPTA effect⁵.
- **Coupled flash photolysis-induced transitions** of an intracellular caged Ca²⁺ buffer that acutely release Ca²⁺, produce instantaneous increases in SANC AP firing rate³.
- **Coupled small fluctuations each cycle:** Slight intrinsic variation in steady-state cycle length is also predicted by small stochastic variations in LCR period during DD⁶.
- **Coupled minute-by-minute changes** in the LCR period during the transition from one steady state to another, for example GPCR activation or phosphodiesterase inhibition, or from disabling SR Ca²⁺ cycling predict concomitant changes in AP cycle length (review¹).
- **Coupled function in different steady states** (Fig.2B,C)

Symmetry of clocks' interactions in the context of numerical modeling

Each piece of experimental evidence for the coupled-clock system (discussed above) is quantitatively simulated by a novel numerical model⁷. Fig.3E, for example, illustrates the model prediction of coupled transitions following voltage-clamp removal. The unique feature of this model is that Ca²⁺ release is controlled by [Ca²⁺] in SR and the submembrane space; SR Ca²⁺ loading, in turn, is determined by components of both clocks, including SERCA, RyR, LCCh, NCX, etc. Classical numerical models of pacemaker cell function, even those describing SR Ca²⁺ release function, fail to reproduce the experimental evidence described, because cell Ca²⁺ dynamics is not correctly formulated in these models.

Experimental evidence indicates that (as in a novel numerical Maltsev-Lakatta model⁷) the correct description of intracellular Ca^{2+} cycling requires that the SR Ca^{2+} release increases two times per cycle: 1) during AP (as Ca^{2+} transient) and 2) during DD (as LCRs forming LDCaE). However, the crucial LCR/LDCaE phase is lacking in classical models, including Dokos, Zhang, and Kurata models, as well as Noble-Noble 1984 model (Maltsev-Lakatta, unpublished results). Individual rhythmic LCRs have been numerically modeled within a complex system of interacting Ca^{2+} release units in SANC⁸.

The roles of I_f and I_{NCX} in a coupled clock pacemaker system

The two inward currents ($I_f + I_{\text{NCX}}$) and decaying I_K form the MDP and initiate DD (Fig.1D). Like I_f , I_{NCX} is also activated by AP repolarization and, hence, I_{NCX} is also a “hyperpolarization-activated” current! In contrast to I_f , however, I_{NCX} activates almost instantaneously (I_f activation kinetics are rather slow of ~ 1 s). Being both voltage- and Ca^{2+} -dependent, I_{NCX} is additionally strongly activated by intracellular Ca^{2+} transient during AP repolarization (Phase-I, Fig.1D). Recent studies utilizing a fast 2D-camera indicate that LCRs actually begin to emerge at the time of MDP and early DD, i.e. concomitantly with I_f ⁶. While during DD I_f reverses, inward I_{NCX} becomes strongly further activated by growing LCRs (Phase-II, Fig.1D) prompting/igniting next AP. Finally, Ca^{2+} transient during AP-upstroke (before overshoot) generates Phase-III of I_{NCX} -activation.

Since pharmacologic maneuvers or genetic manipulations to inhibit I_f do, indeed, produce modest rate reduction in the basal AP firing rate, including a modest reduction of heart rate in humans (review¹), perhaps most important role of I_f is to prevent bradycardia. In other terms, I_f is a “defensive” mechanism rather than a major operant that controls the heart rate. A broad parametric sensitivity analysis of numerical coupled-clock SANC system, has also pointed to a defensive role for I_f to insure system robustness at lower heart rates (when I_f has sufficient time to activate)⁷. Curiously, pacemaker cells of some species even lack I_f (e.g. bullfrog sinus-venosus).

In summary the intimately intertwined, yin-yang type robustness and flexibility of the heart’s pacemaker system of both intracellular molecules (e.g. SERCA and RyR) and sarcolemmal molecules (e.g. LCCh, NCX, K^+ channels, and even HCN channels) underlie stable normal pacemaker cell automaticity.

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Dynamic interactions of a system of electrogenic and Ca^{2+} cycling molecules **Fig.1.**

A. Line-scan image of LCRs (white arrows) with superimposed spontaneous APs in rabbit SANC. B: Confocally measured LCRs (upper panel) and their temporal average (lower panel), i.e. LDCaE that precedes AP-induced Ca^{2+} transient (from¹). C: LDCaE in single SANC of rabbit and guinea-pig (modified from Lakatta et al, Circ Res 2003;92:e45-50 and Rigg et al. Cardiovasc Res 2000;48:254-264, respectively). D: Novel numerical model predicts the yin-yang type interactions within the coupled-clock system of SANC (modified from⁷).

Fig.2.

A: Schematic illustration of interactions of molecules comprising the full coupled-pacemaker clock. Note that the same regulatory factors (red lettering) of the SR Ca^{2+} -clock (gray intracellular area, black lettering) couple the Ca^{2+} clock to the M clock (blue membrane area, blue lettering). G protein-coupled receptors (green lettering) regulate both the Ca^{2+} clock and membrane clock via those same coupling factors which are crucial nodes within the system. B: PKA-dependent changes in phosphorylation of PLB predict changes in LCR period. C: Changes in the steady state LCR period in response to numerous perturbations of the system predict concomitant changes in the steady state spontaneous cycle length. From¹ (updated with data from Vinogradova et al. Circ Res 2010;107:767-775).

Fig.3.

A-C: Rhythmic occurrence of LCRs (white triangles) ceases when SANC is voltage-clamped (VC) at the MDP [A, Recordings of APs in a patch-perforated representative SANC; B, simultaneous linescan image of Ca^{2+} signal (fluo-3); C, Normalized subsarcolemmal fluorescence (averaged within double-headed arrow in B)]. D(i): An example of coupled transition of M-clock and Ca^{2+} -clock following removal of VC (-63mV) to rhythmic beating. Rhythmic LCR activity (white triangles) progressively grows and heralds a progressive increase in AP firing rate. D(ii): Average data on the dynamics of cycle length and LCR signal mass (product of LCR size, duration, and amplitude) after VC removal. Modified from Vinogradova et al. (Circ Res 2004;94:802-809). B: Simulations of a new SANC model⁷ (From Maltsev et al. Chapter#4: Heart Rate and Rhythm. Eds:Tripathi et al. Springer-Verlag Berlin Heidelberg, 2011)