5 Cardiac Pacemaking Is an Emergent Property of Complex Synchronized Signaling on Multiple Scales

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Abstract

The generation of automaticity in the sinoatrial node is a multiscale process involving the integration of complex biochemical and biophysical processes, including a membrane clock and a calcium clock operating in a coupled-clock system within single cells and further complex integration of heterogeneous intercellular signaling at the tissue level. Our current understanding of these processes is reviewed in detail. Ground-breaking recent studies in intact SAN have discovered a new paradigm of SAN operation, and this, along with other frontiers in pacemaker research, are addressed.

Keywords

Pacemaking · Calcium · Ion channels · Synchronization · Ignition · Automaticity · Autonomic · Emergence

5.1 Introduction

Intrinsic cardiac automaticity, or "pacemaking," is the fundamental physiological process that sustains zoological life on Earth. The absence of cardiac automaticity is not compatible with life, save for in very few medically supported circumstances (e.g. cardiopulmonary bypass, ventricular assist devices, ECMO). Substantial increases in our understanding of cardiac automaticity have been made over the last century [1], yet significant uncertainties remain, and paradigms continue to shift [2]. Pacemaker cells that lead cardiac automaticity are located in the sinoatrial node (SAN) of the right atrium and spontaneously generate rhythmic changes of their membrane potential, producing relatively periodic (but not metronomic) spontaneous action potential (APs), so-called "sinus rhythm." The essence of cardiac automaticity is diastolic depolarization (DD)—a slow, spontaneous increase in membrane potential toward the membrane excitation threshold, followed by the firing of an all-or-nothing AP.

The initial focus of pacemaker cell research was on sarcolemmal ion currents (review [3]). In

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silico reconstruction of the ensemble behavior of these currents based on experimental voltage-clamp data is able to generate spontaneous APs, and ultimately the coordinated behavior of these ion channels leading to SAN cell automaticity was dubbed the "membrane clock" (M-clock).

It was with some difficulty that the important role of intracellular Ca cycling in normal automaticity was added to the paradigm. The SAN cell's major intracellular store of Ca is the sarcoplasmic reticulum (SR), which was discovered to generate roughly periodic diastolic Ca releases, dubbed the "Ca clock" (review [4]), whether the cell membrane was present or not. This latterly elucidated clock can impact the M-clock at many "nodes" of interaction between the two, perhaps most importantly through the acceleration of DD via the electrogenic membrane-bound Na/Ca exchanger (NCX) [5, 6]. Further studies suggested that the Ca clock depends on the membrane clock and vice versa, and the functions of both clocks are tightly and synergistically integrated to formulate a coupled oscillator, leading to the coupled-clock theory of normal automaticity [7, 8]. Having coupled clocks ensures both robustness (i.e., "fail safe" operation) and flexibility (i.e., the ability to react to demands for faster or slower AP firing rate) in SAN cells. The coupled-clock paradigm has been intricately numerically modeled [7, 9–11] and extensively reviewed [1, 8, 12]. In this chapter, we review key and novel aspects of this concept. Of particular interest will be challenging the present perspective that SAN output is generated by the collective behavior of loosely coupled SAN cells and is not dominated by the DD of a single cell. Further, the interactive network of mechanisms intrinsic and extrinsic to SAN cells must also interpret and react to signals arising extrinsic to the cell, e.g., stretch, electrotonic impulses, or neurotransmitter or hormonal stimulation of surface membrane receptors. This must result from the timely integration of signaling events at multiple levels within the SAN, including subcellular, cellular (surface membrane), and tissue architecture. This is presently poorly understood, yet extremely important, and represents the current and future frontier of cardiac pacemaker research [13–15].

5.2 The Membrane Clock

Every pacemaker cycle involves the interaction of Ca and membrane clocks via multiple time-, voltage-, and Ca-dependencies of proteins comprising the system. The membrane clock (Fig. 5.1, top) features ion channels, transporters, and pumps. Its key molecules include L-type Ca channels (LCCh) and T-type Ca channels (generating I_{CaL} and I_{CaT}, respectively); K channels; nonselective hyperpolarization-activated cation channels (HCN4), generating I_f; and NCX, generating I_{NCX}. NCX is electrogenic—it exchanges 3Na for 1Ca, thereby generating a membrane current, and this operation is both Ca- and voltage-dependent, executing an efficient coupling mechanism between the clocks.

5.3 The Calcium Clock

The Ca clock (Fig. 5.1, bottom) features the SR refilling Ca pump SERCA, and Ca release channels, ryanodine receptors (RyRs), the latter of which generate rhythmic, diastolic local Ca releases (LCRs). LCRs first appear as small Ca spark-like events early in DD, around the time of the maximum diastolic potential (MDP). As membrane depolarization proceeds, LCRs increase in size and develop local propagation attributes (abrupted waves). In late DD, these propagating LCRs merge into the AP-induced whole-cell Ca transient [16]. The LCR period is the period from the peak of the prior AP-induced cytosolic Ca transient and subsequent diastolic LCR emergence. LCR periods inform not only on the RyR activation but also on the kinetics of recharging the SR Ca capacitor [17].

5.4 Clock Coupling During Diastolic Depolarization

Each spontaneous AP is followed by repolarization and the re-establishment of an MDP, from which DD begins due to the removal of K channel

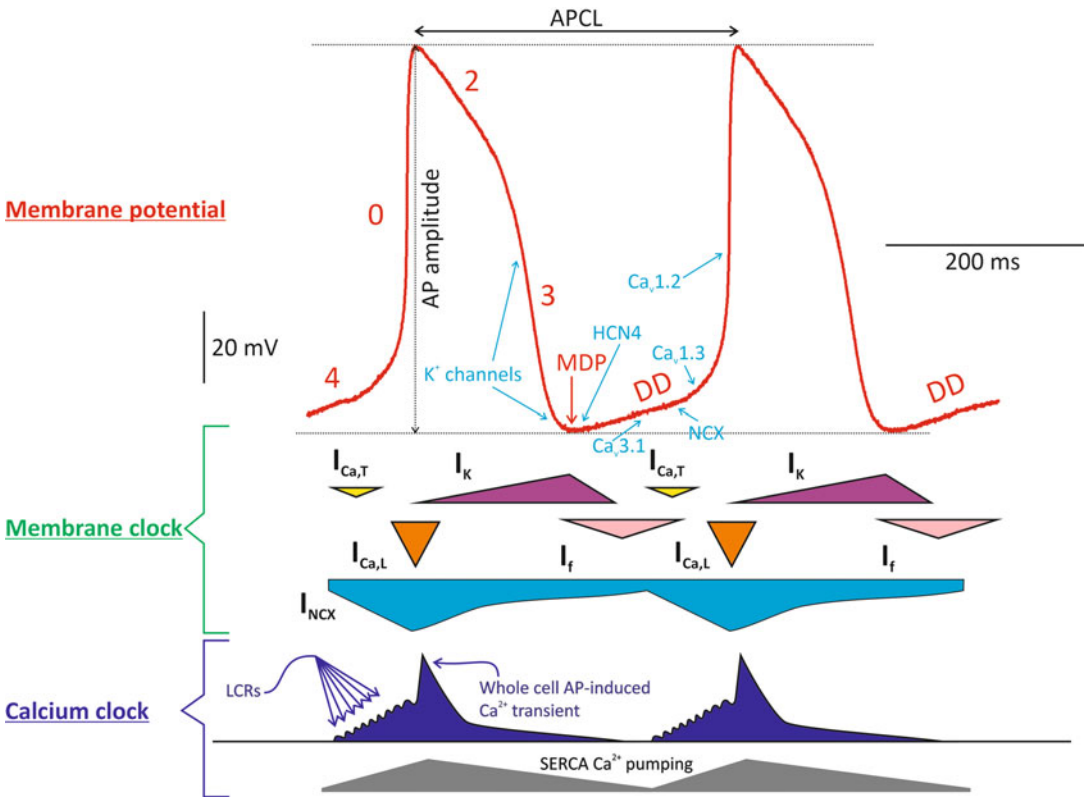

Fig. 5.1 The biophysical "engine" of the coupled-clock system

activation, activation of I_f and I_{CaT}, and spontaneous local RyR activation generating multiple submembrane LCRs. Summation of individual LCRs promoted in part by Ca-induced-Ca-release (CICR) [18, 19] produces an ensemble Ca signal that activates electrogenic inward I_{NCX}. Blooming of the LCR ensemble Ca signal, with associated accelerating I_{NCX} activation, initiates an acceleration in DD to the so-called nonlinear phase [20], activating I_{CaL}. The time from MDP to the nonlinear DD informs on the kinetics of LCR generation and synchronization. I_f decreases during late DD as it gets closer to its reversal potential (near –30 mV).

The relative importance of events in early DD continues to be debated. Some believe that LCRs are the key events that initiate APs, based on several fundamental observations, including (1) spontaneous LCRs occur under voltage clamp [21], independent of membrane potential change, (2) LCRs occur as early as AP repolarization reaches the MDP [16], and (3) oscillatory LCRs are observed in membrane-free (i.e., detergent-permeabilized, "skinned") SAN cells bathed in a physiological [Ca] of 100 nM [21, 22]. These membrane-independent LCRs are generated at rates of 1–5 Hz, i.e., similar to the rates of spontaneous AP firing in SAN cells (in rabbits).

Others favor the importance of low voltage-activated Ca currents, such as I_{CaT} [23] and I_{CaL} generated by Cav1.3 channels ($I_{Cav1.3}$) [24] as primary AP-initiating events. Loss of Cav1.3 function in humans is associated with bradycardia and congenital deafness [25], and Cav1.3 knockout mice have substantially reduced SAN cell Ca dynamics [26].

5.4.1 The Concept of Ignition

A unifying view of the AP ignition process [27] suggests that pacemaker APs are ignited during DD via a feed-forward mechanism that includes membrane potential, LCRs, NCX, I_{CaT}, and I_{CaL}. This ignition phase begins when the magnitude of inward I_{NCX} begins to increase due to its activation by LCRs. I_{NCX}, together with I_f and I_{CaT} depolarizes the membrane potential to the $I_{Cav1.3}$ activation threshold (near –55 mV). During the ignition phase, $I_{Cav1.3}$ depolarizes cell membrane, and concurrently its mediated Ca influx generates more LCRs via CICR that further activates inward I_{NCX}, implementing a feed-forward control that drives membrane potential to the threshold potential of –40 mV required for activation of I_{CaL} via Cav1.2 channels ($I_{Cav1.2}$) that generates the AP upstroke.

Because Ca and membrane mechanisms are so tightly linked within the coupled-clock system, perturbations in either clock inevitably affect the other to some degree and together affect the ultimate readout of the coupled system, which is the AP firing rate. LCR period and AP cycle length (APCL) are similarly affected by either selective perturbation of the membrane clock (inhibition of I_f by ivabradine) or Ca clock (inhibition of SERCA by cyclopiazonic acid) [28], indicating that the LCR period reports the crosstalk between the clocks. In the case of ivabradine-induced bradycardia, the initial effect on membrane clock funny channels reduces the rate of I_{CaL} activation and respective Ca influx and SR Ca loading. Within the novel concept of AP ignition, whereby LCRs interact with NCX and I_{CaL}, this important result can be re-cast in terms of a "time-to-ignition" phase (when I_{NCX} becomes activated by LCRs), determined by coupled-clock interactions, playing a key role in determining cycle length.

Robustness is rigidly built into AP ignition, as one might expect of such a physiologically critical process—genetically modified mice with knock-down of as much as 80% of NCX molecules have normal resting heart rates [29]. This is not evidence of a lack of importance of NCX; instead, numerical modeling of the coupled-clock system revealed that having as few as 20% NCX molecules is sufficient to keep diastolic I_{NCX} amplitude almost unchanged and the ignition mechanism remaining intact [30]. This is thought to be because, under normal function, LCRs occur only in a small part of the cell, leaving a substantial portion of NCX molecules not yet activated by the LCRs. Those NCX molecules in the LCR-free areas represent a substantial depolarization reserve. NCX-deficient cells exhibit a compensatory increase in the spatial extent of the LCR ensemble to activate almost all remaining NCX molecules. However, such baseline compensation comes at a cost: nearly 100% NCX activation at rest leaves no reserve to support a further I_{NCX} increase, hence no ability to increase the AP firing rate in the fight-or-flight reflex [29].

5.5 The AP Upstroke, Plateau, and Repolarization

The AP upstroke relies on the availability of LCCh (Cav1.2 type). More available channels to respond to a membrane voltage change lead to a greater rate of increase (dV/dt) of the AP upstroke. The resultant Ca influx via activated I_{CaL} partially binds to SR-based RyRs and synchronizes their activation via CICR, resulting in synchronous RyR Ca release to generate the whole cell-engulfing AP-induced Ca transient [31, 32]. This partially depletes Ca in the SR. LCCh also begins to inactivate with time at the depolarized membrane potential. The Ca transient (intracellularly derived Ca) and Ca influx via LCCh (extracellularly derived Ca) activates SERCA2 to pump cytoplasmic Ca into the SR, with the rapid (Ca-dependent) kinetics of this process facilitating efficient and timely replenishment of the Ca store.

Membrane depolarization activates K channels and inactivates forward mode NCX, leading to AP repolarization, whose kinetics inform on the

combined actions of K channels, cytosolic [Ca], I_{NCX}, and I_f. K channel activation repolarizes the surface membrane, reactivating forward Na-Ca exchange, which assists in removing the Ca released via the AP-induced Ca release via RyRs. This same Ca also activates repolarizing K channels [33].

Membrane ion channels and transporters regulate the Ca balance of the coupled system not only directly (Ca influx/efflux via LCCh/NCX) but also indirectly, via I_K activation, which repolarizes the membrane and, in doing so, limits Ca influx via LCCh while simultaneously increasing Ca efflux via voltage-dependent activation of forward mode NCX. Even I_f activation indirectly regulates the cell Ca balance by limiting the MDP, thereby limiting voltage-dependent Ca efflux via NCX.

5.6 Intracellular Na Is an Important Modulator of Clock Coupling

The critical role of NCX within the coupled-clock system would naturally suggest a key regulating role for intracellular Na. In SAN cells perturbed by digitonin (blocker of Na/K ATPase, causing intracellular Na accumulation) [34], increases in $[Na]_i$ and $[Ca]_i$ and respective reductions in E_{Na} and E_{Ca} lead to a small reduction in MDP, enhanced LCRs, increased diastolic I_{NCX} and reduced average APCL. As $[Na]_i$ and $[Ca]_i$ continue to increase, MDP, E_{Na}, and E_{Ca} are further reduced; LCR signal becomes reduced, diastolic I_{NCX} decreases, and APCL progressively prolongs, accompanied by increased APCL variability. Numerical modeling reveals that increased $[Na]_i$ causes E_{Na} and E_{Ca} to reduce monotonically and decrease important ionic currents (I_{CaL}, I_{CaT}, I_f, I_{Kr}, and I_{bNa}). Digitonin's ability via a monotonic Na increase to initially increase rate and rhythmicity, followed by a decrease in rate and rhythmicity, followed ultimately by frank arrhythmia, is clear evidence of the fundamental role played by intracellular Na and Ca in the genesis and maintenance of normal heart rhythm (see Fig. 3 in [34]).

5.7 Diastolic Depolarization May Be Regarded as a Phase of Transition

While the membrane clock operates as a limit cycle oscillator, the Ca-clock operates by a completely different mechanism called "criticality" [15]. Ca release channels—RyRs—are organized into and operate in clusters, known as Ca release units in all cardiac cells, including in SAN cells [19]. In vitro experiments and numerical modeling have demonstrated that LCRs emerge via self-organized synchronized activation of Ca release units (driven by explosive CICR), leading to oscillatory, phase-like transitions in SAN cells [18], reinforced in more recent theoretical studies [35, 36] that demonstrate phase transitions (including order-disorder transitions), pointing to the fractal-like functional and structural (hierarchical) organization of RyRs in SAN cells (i.e., within and among Ca release units).

Based on the results of these modeling experiments and the importance of Na (described above), the ignition theory and local interactions during DD can now be viewed in terms of phase transition, criticality, and electrochemical gradient oscillations. As already noted, LCRs begin to occur around the time of the MDP [16], sometimes before. Such early release events are small and stochastic (i.e., disordered), akin to Ca sparks in resting ventricular myocytes. The ensuing simultaneous growth of the diastolic ensemble LCR signal and DD during AP ignition can be viewed as self-organization of the LCRs, interacting with an excitable cell membrane that reaches criticality in late diastole, followed by a phase transition manifest as the rapid AP upstroke that triggers a global systolic Ca transient.

5.8 Emerging Players in the Coupled-Clock System

Several new and relatively unclear mechanisms are increasingly believed to be important in the genesis and maintenance of SAN automaticity.

Store-operated Ca entry (SOCE) via *store-operated Ca channels* may represent an additional pathway for Ca entry into the SR upon Ca store depletion. Murine SAN experiments suggested that store-operated activity was attributable to TRPC expression and that store-operated Ca channels may be involved in regulating pacemaker firing rate [37]. More recently [38], two new proteins, stromal interacting molecule (STIM) (an endoplasmic reticulum Ca sensor) and Orai (surface membrane channel) were implicated in SOCE in pacemaker cells; after store depletion, STIM1 redistributed to the cell periphery and co-localized with surface membrane-linked Orai1, suggesting the involvement of these proteins in SOCE activity and cardiac pacemaker function. The TRPC3 channel is also involved in the SOCE mechanism, and TRPC3(–/–) mice experience sinoatrial arrhythmias [39].

The *inositol 1,4,5-trisphosphate receptor* (IP3R) represents a second mechanism of Ca release from the SR and contributes to the automaticity of SAN cells: pharmacological perturbation of these channels affected automaticity in wild-type mice, but not in transgenic IP3R2 knockout mice [40]. Furthermore, stimulation of IP3Rs accelerates spontaneous beating rate in isolated mouse SAN cells, while inhibition slows the rate [41], and in mice with uncoupled clocks (i.e., in atria-specific NCX knockout mice), IP3R agonists and antagonists modulated the rate of spontaneous Ca waves, suggesting that IP3R-mediated SAN pacemaker regulation is controlled primarily by the Ca clock rather than the membrane clock [41].

SK4 *Ca-activated K channels* generate $I_{K(Ca)}$—inhibition of this current in spontaneously beating human embryonic stem cells depolarized the MDP and suppressed automaticity [42]. All three SK isoforms (SK1, SK2, and SK3) have since been identified in mouse SAN [33]. Inhibition of SK channels with apamin (i) prolonged APs in isolated SAN cells, (ii) slowed diastolic depolarization, and (iii) reduced pacemaker rate in isolated SAN cells and intact SAN tissue. It is proposed that these channels modulate pacemaking via activating a repolarizing Ca-activated current. Intraperitoneal injection of SK4 channel blockers greatly reduced the arrhythmias in CASQ2-D307H knock-in mice and CASQ2 knockout mice at rest and during exercise, demonstrating a role of SK4 channels in adult pacemaker function and suggesting that these channels may be therapeutic targets for the treatment of certain cardiac arrhythmias [43].

5.9 Biochemical Characteristics of the Coupled-Clock System

5.9.1 Enhanced Basal Levels of cAMP and Protein Phosphorylation by PKA and CaMKII Drive SAN Automaticity

Uniquely among cardiac cells, SAN cells express neuronal, Ca-activated adenylate cyclase (AC) types AC1 and AC8 [44, 45]. Under baseline conditions, SAN Ca binds to calmodulin to activate these ACs, leading to a high basal level of cAMP-mediated, protein kinase A (PKA)-dependent phosphorylation of coupled-clock proteins [22, 45, 46].

The critical functional importance of these Ca-dependent ACs has been validated in studies of genetically modified cells and animals. In vivo adenoviral implantation and expression of AC1 into the left bundle branch of dogs with heart block results in highly efficient heart automaticity emanating from the implant site [47]. Along similar lines, drug-induced activation of the AC8 gene substantially increases rate and improves rhythmicity in cardiac pacemaker-like cells differentiated from the genetically modified P19 cell line [48]. Recent studies [49] have also shown that increased cAMP production in mice with heart-targeted expression of AC8 (TG^{AC8}) is accompanied by a marked increase in heart rate with a concurrent reduction in heart rate variability (discussed below), regardless of autonomic innervation.

The challenge of directly measuring PKA activity in SAN cells was eventually met in cultured rabbit SAN cells infected with an adenovirus expressing the FRET sensor AKAR3

[50]. Here, the kinetics and stoichiometry of increased PKA activity matched the increase in AP firing rate in response to β-adrenergic receptor (β-AR) stimulation or phosphodiesterase inhibition. Associated numerical modeling predicted that phospholamban phosphorylation is a potent PKA target to stimulate positive chronotropism.

Phosphorylation of coupled-clock proteins is also achieved via Ca/calmodulin-dependent protein kinase II (CaMKII) [51, 52], which (like AC and LCRs) is highly localized beneath the surface membrane [53]. Mice genetically lacking CaMKII fail to mount a fight-or-flight SAN response [54]. However, experiments in isolated SAN cells subsequently showed twofold greater basal levels of activated (autophosphorylated) CaMKII in SAN cells compared to ventricular myocytes; this high basal CaMKII activation modifies the phosphorylation state of phospholamban, RyR, LCCh (and likely others), affecting LCR period and other LCR characteristics, and ultimately regulates both normal and reserve cardiac pacemaker function [51].

5.9.2 Pumping the Brakes: Mechanisms Intrinsic to the Coupled-Clock System That Restrain Its Basal AP Firing Rate

The coupled-clock system would be perpetually self-amplifying until maximal function was reached (i.e., Ca release begets more Ca release via PKA and CaMKII pathways (Fig. 5.2, green arrows)) were it not for continuous and concurrent mechanisms functioning to drive down SAN cAMP level and phosphorylation levels (Fig. 5.2, red arrows). High basal phosphodiesterase (PDE) activity is one such restraining mechanism [55]. The cAMP-degrading PDE1, PDE3, and PDE4 represent major PDE activities in rabbit SAN cells, with several specific targets (LCCh and phospholamban) being regulated by basal concurrent PDE3 + PDE4 activation [56]. Basal cardiac pacemaker function regulated by concurrent PDE3+PDE4 activation operates in a synergistic manner via a decrease in cAMP/PKA phosphorylation, suppression of LCR activity, and prolongation of the LCR period and APCL [57]. High basal phosphoprotein phosphatase activity also operates in SAN cells [58] to limit PKA and CaMKII-dependent phosphorylation. Other restraining mechanisms include the limitation of Ca influx and cell Ca load by calmodulin-mediated LCCh inactivation.

The net result of these restraining mechanisms is that the cAMP production, protein phosphorylation, basal LCR period, and APCL are maintained near the midpoint of their functional ranges. Compare this with ventricular myocytes, where the basal phosphorylation of Ca-cycling proteins is low to prevent undesirable and potentially arrhythmogenic Ca waves. Forced phosphorylation of SR Ca-cycling proteins in a physiologic Ca milieu unleashes a high-power, rhythmic Ca clock in ventricular myocytes generating LCRs and spontaneous APs, generating a SAN cell phenotype from the ventricular myocyte [60]. These results demonstrated that all cardiomyocytes have the potential for automaticity in the right circumstances but that normal cardiac function relies on suppression of this phenotype in the working myocytes of the atria and ventricles.

5.10 Coupling Biochemical and Biophysical Aspects of the System Yields Functional Flexibility

5.10.1 High Basal Levels of cAMP and Phosphorylation Translate into Powerful LCR Signals

The biochemical and biophysical aspects of the coupled-clock system are tightly linked—spontaneous LCRs are more likely to occur in response to a higher SR Ca load, which is determined by the Ca available for pumping into the SR and the phosphorylation status of the Ca-cycling proteins,

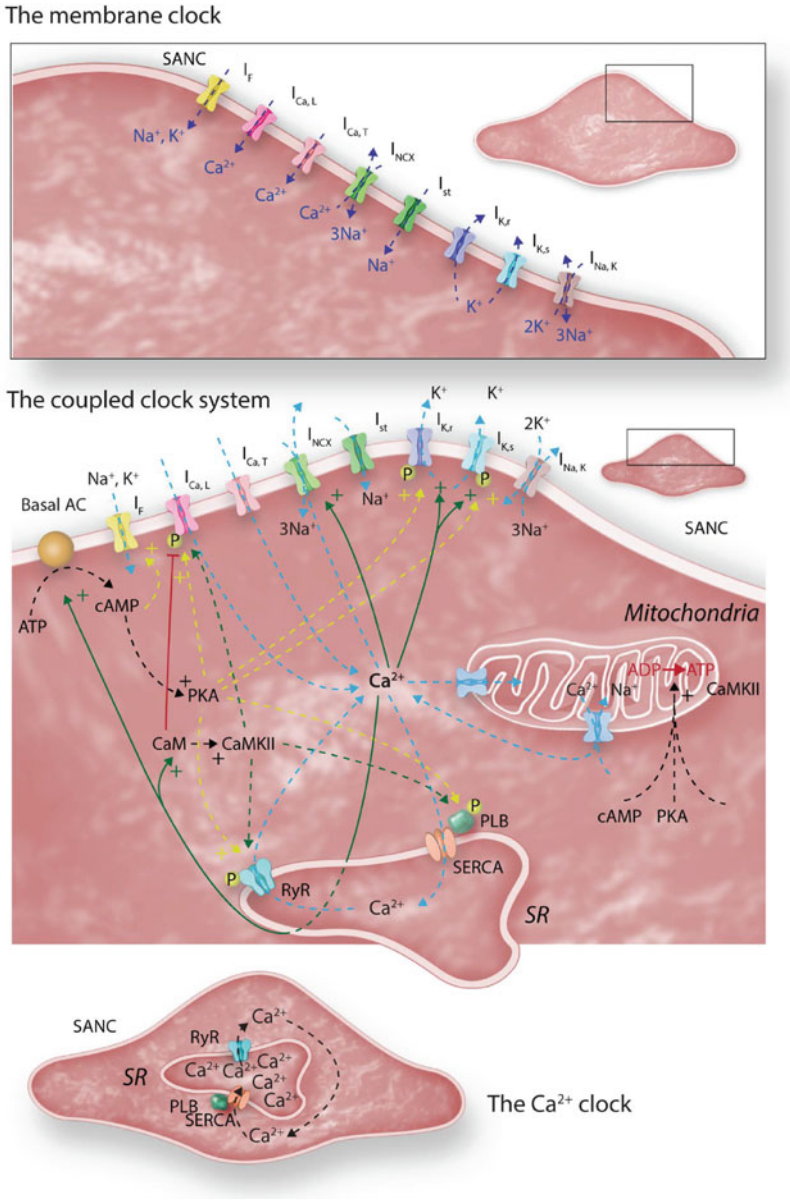

Fig. 5.2 The biochemical "engine" of the coupled-oscillator system (from [59])

including phospholamban (major regulator of SERCA activity), RyR, and LCCh. SR Ca refilling kinetics regulate the LCR period and spontaneous beating rate of rabbit SAN cells [17]. PKA- and CaMKII-dependent phosphorylation of Ca-cycling proteins and LCCh synchronizes spontaneous RyR activation. The restitution process that determines the LCR period is regulated by (1) SR Ca-cycling kinetics, i.e., rate of Ca pumping into the SR; and (2) the threshold of SR Ca load required for spontaneous RyR activation. Exquisitely biophysically detailed numeric models support these findings [7, 9, 19, 50, 61].

5.10.2 Autonomic Modulation of AP Firing Rate Occurs via the Same Coupled Biophysical and Biochemical Mechanisms

The coupled-clock system has to function flexibly over the range of rates necessary to sustain life. It does this via variation in G protein-coupled receptor signaling to link both adrenergic and cholinergic receptors (ChR) to the very same critical components of the coupled-clock system that regulate the basal state LCR period [8]. Stimulation of sympathetic β-ARs in SAN cells increases the spontaneous AP firing rate via G_s and further downstream signaling components resulting in a multitude of effects on proteins of both clocks, including ion channels of the membrane clock [3], specifically I_K and I_{CaL} (via PKA-dependent phosphorylation) and I_f (via a direct effect of cAMP). Note that the direct effect of cAMP on I_f has recently been studied in exquisite detail using a knock-in mouse expressing cAMP-insensitive HCN4 channels—these mice showed sinus dysrhythmia, marked sinus bradycardia, sinus pauses, and an inability to increase heart rate appropriately ("chronotropic incompetence") [62].

β-AR stimulation similarly affects the Ca clock, via changes in cAMP/PKA-dependent phosphorylation due to AC activation and phosphatase inhibition, leading to altered SR Ca release via RyR, and Ca sequestration via SERCA [63–65]. The ultimate effect is the generation of stronger and earlier LCR ensemble signals [17, 22, 65], activating stronger and earlier I_{NCX}, accelerating DD toward threshold potential [9]. In addition, CaMKII-dependent phosphorylation of phospholamban and RyR also increases in response to β-AR stimulation (likely, secondary due to changes in cell Ca dynamics and concomitant calmodulin activation, affected primarily by PKA activation). Thus, CaMKII-dependent phosphorylation has an important role as a Ca-calmodulin-dependent "late" amplifier of the initial effect of β-AR stimulation.

ChRs contrastingly acts as a physiological "brake" on heart rate. ChR signaling activates G_i proteins that couple to several downstream targets: (1) G_i-βγ activation of I_{KACh} (see review [3] for details) ; (2) a G_i-α_s coupled reduction in AC activity that limits cAMP/PKA-dependent phosphorylation. Ultimately, in contrast to β-AR stimulation, ChR stimulation decreases the Ca balance of the system, which leads to a reduction in the AP firing rate.

5.10.3 Mitochondria Provide Tailoring of the "Gas" to Fuel the System

Mitochondrial ATP use is unique in SAN cells [66], most being consumed in the synthesis of cAMP and Ca cycling maintenance (rather than in myofilament contraction as in working myocardium) (Fig. 5.2). That cyanide reduces intracellular Ca transient amplitude and AP firing rate in primary pacemaker cells [66, 67] suggests mitochondria have an important role in pacemaking. There is evidence that the Ca-activated-cAMP/PKA signaling cascade not only drives basal ATP consumption but also regulates ATP production [66]. cAMP/PKA signaling can affect ATP production by phosphorylation of several mitochondrial proteins (for review, see [68]), and a decrease in basal cAMP/PKA signaling in SAN cells appears to signal to mitochondria to reduce ATP production. Crosstalk occurs between the SR and the mitochondria through the currency of Ca [69], for example, an increase in mitochondrial Ca by inhibition of mitochondrial NCX decreases the SR Ca load and reduces the ensemble LCR Ca signal. In contrast, a reduction in mitochondrial Ca by inhibition of mitochondrial uniporter increases the SR Ca load and increases the ensemble LCR Ca signal.

5.10.4 Local Signaling in Microdomains

Complex crosstalk of biophysical and biochemical aspects of the coupled system described above is precisely organized and implemented in time

and space within subcellular microdomains associated with discrete clusters of different ion channels, transporters, and regulatory receptors (review [70]). Such subcellular microdomains represent an interacting network of various proteins that function locally and efficiently as macromolecular signaling complexes. The spatial organization of essential signaling components makes it possible for different proteins to effectively interact and be tightly controlled by a variety of neurohormonal signaling mechanisms so that a limited pool of protein types and second messengers can generate a large variety of cellular responses commensurate to the current physiological demand. As an example of such local regulation, co-localization of PDE3 and PDE4 beneath the sarcolemma or in striated patterns inside SAN cells strongly suggests that PDE-dependent regulation of cAMP/PKA signaling is locally executed [56].

5.11 The Human Coupled-Clock System

Our historical appreciation of the coupled-clock operational paradigm stems largely from studies in small mammal hearts. Few studies exist focusing on pacemaker cell function in human hearts [71], and until very recently, none had substantiated the coupled-clock theory in human hearts. Tsutsui et al. [72] made this breakthrough, simultaneously measuring LCRs and membrane potential in freshly isolated human SAN cells and demonstrating all of the salient features of the coupled clock previously only seen in experimental animals.

5.11.1 The Workings of Automaticity Are Finely Tuned to Satisfy Physiological Demand in Individual Species

It is well-recognized that major species differences in heart rate exist. For example, baseline APCL in mice is ~100 ms, while in humans is ~1000 ms. The work of Tsutsui et al. referred to in the above paragraph, broadly showed that characteristics of the membrane and calcium clock are similar among mammalian species, including humans [72]. What has remained unclear is the degree and nature of differences in the coupled clock between species. Recent intricate work from Tagirova et al. has demonstrated that parametric Ca and membrane potential kinetic transitions in APCL in SAN cells in vitro, heart rate in vivo, and body mass can be scaled, and demonstrate self-similarity (i.e. obey power laws) across species [73]. They concluded that "In designing optimal heart rate to match widely different body mass and energy requirements from mice to humans, nature did not 'reinvent pacemaker cell wheels', but differentially scaled kinetics of gears that regulate the rates at which the 'wheels spin'." This work represents a frontier in our current trans-species understanding of automaticity and paves the way for the future development of novel therapies and biological pacemakers (see Sect. 5.16 concerning future studies).

5.12 AP Firing Rate and Rhythm Reflect Clock Coupling Determined by Clock Protein Phosphorylation Status

5.12.1 Beat-to-Beat Ca-Dependent Regulation of the Coupled-Clock System

A hallmark of the SAN automaticity is AP firing rate variability, i.e., beat-to-beat changes in spontaneous AP cycle length. Initially, this variability was attributed to the stochastic opening of sarcolemmal ion channels, mainly I_{CaL} and I_K [74]. Latterly, the spotlight fell on the Ca clock, specifically whether Ca signals variably influence the duration of each cycle on a beat-to-beat basis. Vinogradova et al. [21] showed that the LCR period and LCR signal mass remain strongly coupled to APCL, not only in the steady-state AP firing but also in each cycle during the transient state after the removal of the voltage clamp at the MDP. Further experiments clearly demonstrated

beat-to-beat Ca-dependent regulation of APCL: acute Ca release from a caged Ca buffer by flash-induced photolysis or from the SR by low concentrations of caffeine acutely reduced APCL via activation of LCRs and NCX in the same cycle that the perturbation was applied [75, 76].

LCRs are stochastically generated by RyRs and demonstrate clear cycle-to-cycle variations in both individual and meantime of LCR occurrence during DD. Because Ca regulates automaticity on a beat-to-beat basis, it follows that LCRs must contribute to the beat-to-beat variability of spontaneous AP firing. This was proven in experiments using high-speed, high-resolution cameras: while individual LCR timing varied, the LCR period averaged for all LCRs in a given cycle closely predicted the time of occurrence of the next AP [16]. Further studies [77] showed that the changes in the average LCR period and its variability in response to chemical perturbation of the coupled-clock system (with cyclopiazonic acid or ivabradine) are correlated with changes in APCL and APCL variability. The study concluded that the stochasticity within the coupled-clock system affects and is affected by the AP firing rate and rhythm via modulation of the effectiveness of clock coupling.

5.12.2 Mechanisms Affecting Clock Coupling

Mechanisms influencing the coupling of the clocks are of critical importance in understanding the SAN's ability to change its rate/rhythm. Yaniv et al. [77] showed that the gradual increase of APCL variability (along with APCL) produced by increasing concentrations of ivabradine is accompanied by gradual decreases in phospholamban phosphorylation at Ser16, pointing to a key role of PKA-dependent phosphorylation in the clock coupling and APCL variability [77].

Moen et al. [49] used mice with heart-targeted expression of AC8 (TG^{AC8}) to demonstrate that increased AC activity in SAN tissue is accompanied by a marked increase in heart rate and a concurrent marked reduction in heart rate variability, both in the absence or presence of dual autonomic blockade, providing further evidence for the importance of the cAMP-PKA-dependent clock-coupling mechanism. Another important result of this study is that variability in spontaneous beating intervals in isolated SAN tissue and single SAN cells, devoid of autonomic neural input, suggests that clocks (and clocks coupling) intrinsic to SAN cells may also contribute to heart rate and heart rate variability in vivo.

5.12.3 Aging Affects Clock Coupling

The full spectrum of mouse SAN AP firing rates requires PKA-dependent Ca signaling [78], but the fidelity of this signaling and, therefore, clock coupling deteriorates with age. Liu et al. [79] reported that the sensitivity of the SAN beating rate responses to both muscarinic and adrenergic receptor activation becomes decreased in advanced age. The sensitivity of the SAN beating rate to cAMP perturbations is also reduced. These compromised clock functions, including a reduced SR Ca load and reduced size, number, and duration of spontaneous LCRs, coincided with decreased expression of crucial SR Ca-cycling proteins (including SERCA, RyR, and NCX). Heart rate variability is also affected by aging due to reduced clock coupling. In the isolated SAN, the sensitivity of the average beating interval and beating interval variability in response to autonomic receptor stimulation or activation of mechanisms intrinsic to pacemaker cells by an increase in cAMP production (by phosphodiesterase inhibition) declines with advanced age [80].

5.12.4 Autonomic Signaling and System "Memory"

Extrinsic factors affecting SAN cells, including mechanical, autonomic, and hormonal factors, modulate the rate and rhythm of spontaneous AP cycles. Extrinsic adrenergic input via β-AR increases the phosphorylation of clock proteins that increases Ca influx in SAN cells. This

indirectly and directly increases synchronization of diastolic LCR events, accelerating the kinetics of self-organized growth of the LCR ensemble Ca signal and activating NCX and $I_{CaL,}$ which both ignite the AP *almost simultaneously* in time, increasing rhythmicity and decreasing variability [19, 61]. Contrastingly, external cholinergic inputs and associated low phosphorylation levels leave LCRs small and disordered in time and space (like sparks in ventricular cells). Consequently, the peak of the LCR ensemble signal and attendant AP ignition occurs with substantial variability, decreasing the rhythmicity of AP firing. Other important cues to AP firing are generated by additional members of the coupled-clock system (including HCN4, K channels, SERCA, etc.). This highly complex oscillatory system has memory implemented by the SR Ca load level and phosphorylation states of clock molecules that reflects the history of stimulation and frequency changes in previous cycles; and all cues are interrelated and become recursive in such a system [81]. Both phosphorylation and Ca modulation of K channels in response to βAR stimulation accelerate membrane repolarization, which is required for more rapid AP firing to occur. A more frequent AP firing, ipso facto (via increased Ca influx by I_{CaL}) increases SR Ca load, leading to more robust and synchronized spontaneous LCR signals.

5.13 Increasing Clock Coupling: From Dormancy to Highly Rhythmic Firing

SAN cell experiments have traditionally focused on cells beating spontaneously at an arbitrarily defined "physiological" rate. Other healthy appearing isolated cells were ignored. Kim et al. [82] examined *all* single cells isolated from guinea pig SAN, including rhythmically firing, dysrhythmically firing, and cells without any apparent spontaneous firing activity ("dormant"). LCRs are present in all cell types, indicating that SAN cells can generate subthreshold signals that have never previously been considered in the pacemaker operational paradigm. β-AR stimulation increases LCR size and synchronizes LCR occurrences in all dysrhythmic, and about a third of dormant cells, some of which develop automaticity, and LCRs become coupled to spontaneous AP-induced Ca transients. The majority of dysrhythmic cells become rhythmically firing in response to β-AR stimulation. Biophysical measurements combined with numerical model simulations indicate that the two previously unstudied dysrhythmic and dormant cell populations have intrinsically partially or completely uncoupled clocks and that they can be recruited to fire rhythmically through β-AR stimulation via increased rhythmic LCR activity reflecting increased clock coupling.

Similar findings were made in cells isolated from human SAN [72]. Nonbeating arrested human SAN cells, or those that do not generate spontaneous APs, exhibit extreme clock uncoupling, being relatively depolarized yet still continuing to generate LCRs. Such "dormancy" or "arrest" can be linked to the depolarized membrane potential (near –35 mV), with I_{NCX} being closer to its reversal potential. When LCRs fail to activate I_{NCX}, partial uncoupling of the Ca and membrane clocks occurs, resulting in substantially slower, dysrhythmic AP firing or even AP failure vs. optimal clock coupling in rhythmically firing cells. Extreme clock uncoupling leads to failure of spontaneous AP generation, which is once more restored by recoupling of the clocks by β-AR stimulation.

5.13.1 Self-Similarity Is a Key Characteristic of SAN Cell Automaticity

SAN cells do not beat metronomically, that is to say, the intervals between action potentials are not identical; instead, they vary by varying amounts from beat to beat. Autonomic innervation has a large impact on this in vivo, yet the characteristic remains in denervated preparations, i.e., single cells, isolated SAN preparations, and isolated hearts. One consequence of this variability is that SAN cells and their pacemaker

mechanisms never achieve equilibrium during AP firing. But what is the nature of this variability? Yang et al. recently comprehensively studied rabbit SAN cells across the whole range of firing rates affected by autonomic agonists and found that all studied parameters of automaticity (including means and variabilities of APCL, local Ca release kinetics, Ca transient decay times, diastolic depolarization rates, AP repolarization times) demonstrate self-similarity ("concordance") across the physiological spectrum of heart rates [81]. This suggests a hitherto unrecognized degree of protein cooperation and synchronization, whereby each AP represents the most potent integrator/synchronizer of the prior diastolic depolarization and ignition events. This kind of self-similar behavior in pacemaking represents another frontier in our understanding of SAN automaticity and mandates further study.

5.14 Marked Electrophysiological Heterogeneity Exists Among SAN Cells

The importance of cAMP-dependent phosphorylation in clock coupling (or fidelity of coupling) is clear, yet how different degrees of phosphorylation translate in clock coupling and specific cell behaviors (e.g., dormancy) will depend on the expression level of clock proteins in individual cells. This cannot be assumed to be the same from cell to cell—immunocytochemical labeling of the LCCh, NCX, RyR2, and SERCA2 varies widely in SAN cells [83]. SAN cells exhibit a substantial degree of cell-to-cell variability in the functional expression of I_{CaL}, I_f, and I_K [84, 85]. Patch clamp recording of key ion currents in the same cell, relationships between basal beating rate and I_{CaL} and I_f density were found, along with a positive relationship between I_f and I_K; the response to Ca-cycling blockade was also correlated with I_f density [85]. These results demonstrate the existence of SAN cells with substantially different clock-coupling capabilities that could be important to execute specific roles at specific times as dictated by the prevailing physiological milieu.

5.15 The Forefront of SAN Physiology: Time for a New Paradigm?

The SAN exhibits a high degree of structural and functional complexity, and these characteristics seem to be critical for its central role in cardiac physiology—to reliably deliver automaticity, flexibility, and robustness [86]. But how specifically this enormous heterogeneity translates into robust and flexible SAN operation still remains an unsolved mystery of the cardiac pacemaker field. Numerous SAN cell characteristics have been described including diversity in cell shape [85] and protein expression; the arrangement of cells within the SAN tissue (e.g., gradient vs. mosaic [87]); autonomic neuronal input [88]; cell-to-cell electrical and mechanical interactions [89, 90]; and intranodal impulse initiation, spread within, and exit from, the SAN [91–94].

A generalized view of impulse initiation and transmission in the SAN emerged about 40 years ago, in which a dominant or "master" pacemaker cell or leading pacemaker center dictates the excitation rate and rhythm of thousands of other subservient SAN pacemaker cells by overdriving their intrinsic spontaneous excitation rates [91, 95]. Shortly thereafter, the idea of mutual entrainment of coupled oscillators [96] was applied to the coordinated firing of the entire SAN [97, 98], whereby individual SAN cells, loosely connected through low resistance junctions, mutually entrain each other to fire APs with a common period (dubbed the "democratic" process). In this theory, the frequency of impulses that exit the SAN lies somewhere between the fastest and slowest spontaneous intrinsic excitation rates of resident SAN cells, and there can be marked phase differences among spontaneous excitation in individual cells. Rather than mimicking classical electrical conduction by consecutively exciting each other, as in ventricular muscle tissue, according to this theory, SAN cells are instead mutually entrained by phase resetting, and conduction within SAN is only "apparent" [98, 99]. The idea of apparent conduction was supported by later studies that

showed that the cardiac impulse could arise from different locations [100]. Then Verheijck et al. [101] performed studies utilizing computer-controlled coupling conductance between individual pacemaker cells and found a critical coupling conductance for 1:1 frequency entrainment of less than 0.5 nS, which could be generated by a few connexin molecules only.

Classic macroscopic imaging of voltage and Ca signals within intact SAN tissue lack the resolution to characterize LCR events occurring within individual cells within SAN tissue [92]. Even though recent studies at higher optical magnification detected LCRs within individual cells of SAN tissue [102, 103], they did not systematically examine their role in impulse initiation and spread across individual cells within the SAN. Therefore, the current paradigm of SAN impulse generation is that full-scale APs of a common frequency are initiated at one site and conducted within the SAN along smooth isochrones yet does not feature fine details of Ca signaling known to be present in isolated SAN cells.

A ground-breaking recent study by Bychkov et al. [2] investigated subcellular Ca signals within and among cells comprising the SAN tissue. The study combined immunolabeling with a novel technique to detect the occurrence of LCRs and AP-induced Ca transients (APCTs) in individual pixels across the entire mouse SAN. At high magnification, Ca signals appear markedly heterogeneous in space, amplitude, frequency, and phase among cells comprising an $HCN4^{+}$/$CX43^{-}$ cell meshwork. The signaling exhibits several distinguishable patterns of LCR/APCT interactions within and among cells: in some cells, APCT occurrence is preceded by diastolic LCRs occurring in the same cell (i.e., similar to the coupled-clock operation discovered in isolated cells); other cells do not generate spontaneous APCTs, but have subthreshold LCRs only (like in dormant cells, described above), which precede APCT generation by adjacent cells; APCTs of various frequencies are generated steadily or in bursts. Apparently, conducting rhythmic APCTs of the meshwork are transferred to a truly conducting $HCN4^{-}$/$CX43^{+}$ network of striated cells via narrow functional interfaces where different cell types intertwine. At low magnification, the earliest APCT of each cycle occurs within a small area of the HCN4 meshwork, and the subsequent APCT throughout SAN pixels is discontinuous. Similar heterogeneous signaling has also been demonstrated in SAN isolated from human hearts and from genetically manipulated mice (pCAGGS-GCaMP8) in which Ca signals are linked to HCN4 expression [2].

In summary, rhythmic, synchronized APs emerge from heterogeneous, subthreshold Ca signaling not detected in low-resolution macroscopic isochrones. These results require a fundamental paradigm shift in our understanding of SAN function to include complex/multiscale integration of subthreshold Ca signals with full-scale APs (Fig. 5.3), resembling the emergence of organized signals from heterogeneous local signals within neuronal networks, e.g., Bötzinger cells of the brainstem, interstitial cells of Cajal in the gut, and uterine smooth muscle [104–106]. In their associated editorial, Clancy and Santana suggested emergent pacemaking as a complex multiscale process with a possible role of stochastic resonance that can increase the synchronization and robustness of the pacemaking system [14].

Thus, classical SAN pacemaking concepts of driving, pacing, leading, and concentric propagation, associated with biological "dictatorship," should be replaced with concepts of "emergence and heterogeneity." While the idea of the "democratic" process within weakly coupled entrained AP oscillators [97, 98] had challenged the old "dictatorship" concept, it considered full-scale AP firing by pacemaker cells at one common frequency via mutual entrainment. Surprisingly, there is no such thing as "*the* pacemaker cell" within the new paradigm. No cell alone or in a cluster initiates the impulse; rather, numerous cells (individually and in clusters) generate heterogeneous complex (partially periodic) signals, both subthreshold LCR and/or full-scale APs of different frequencies, and those signals integrate and synchronize over the cellular meshwork so that the rhythmic output **emerges** from those

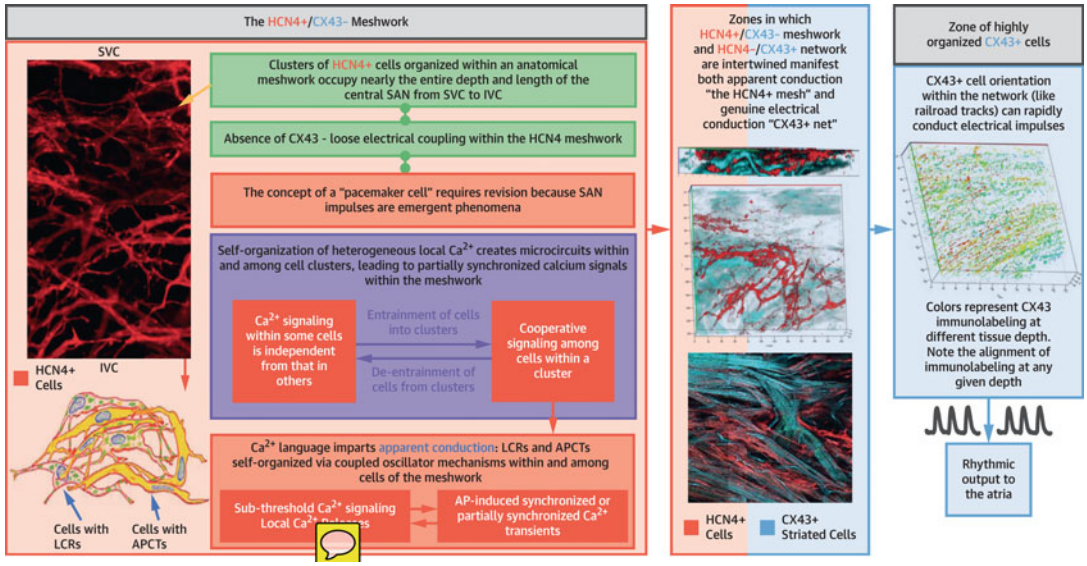

Fig. 5.3 A novel SAN structure/function paradigm. Panels illustrate HCN4 meshwork (left panel), intertwining area (central panel), and CX43 network of cells (right panel). Spontaneous signals are generated within the HCN4 meshwork and transmitted to the CX43 network of cells through the intertwining area. AP, action potential; APCT, action potential-induced Ca^{2+} transient; CX43, connexin 43: HCN4+, hyperpolarization-activated cyclic nucleotide-gated channel 4; IVC, inferior vena cava; LCR, local Ca^{2+} release; SAN, sinoatrial node (from [2]). See also online video 9 from the same paper (https://www.jacc.org/cms/asset/aa14ddc8-c5d5-45d0-bcce-d2c0e36c9651/mmc11.mp4)

multiple signals as the result of this complex signal processing. The recent work by Fenske et al. [62, 107] in knock-in mice expressing cAMP-insensitive HCN4 channels has further underlined the likely importance of nonfiring SAN cells—they showed that tonic and mutual interaction (so-called "tonic entrainment") between firing and nonfiring cells slows down pacemaking in the SAN and that cAMP increases the proportion of firing cells, and in doing so increases rate of automaticity, and increases resistance to vagal signaling.

5.16 Summary, Future Studies, and Harnessing the Coupled-Clock Operation for Clinical Applications

Above, we have reviewed contemporary concepts surrounding the generation of automaticity in the SAN—a multiscale process involving the integration of complex biochemical and biophysical processes. The Ca clock generates diastolic LCRs that are small and disordered at about the time of MDP but then self-organize into larger events that initiate AP ignition. The system reaches *criticality* in late diastole and undergoes a *phase transition* culminating in AP generation. The ignition process involves feed-forward local interactions among LCRs, NCX, and I_{CaL}. Clock coupling is biochemically regulated by a complex enzyme network featuring high basal levels of cAMP (produced by Ca-activated AC1 and AC8) and a high basal degree of clock protein phosphorylation via cAMP-dependent PKA and CaMKII. The feed-forward system is checked by highly constitutionally active PDEs and phosphatases, leaving the engine's basal state tuned to operate near the middle of its spectrum. Various degrees of cAMP production and phosphorylation achieved by autonomic receptor modulation increase or decrease clock coupling and hence AP firing rate and rhythm,

commensurate to the chronotropic physiologic demand. The degree of clock coupling determines the entire spectrum of APCL and APCL variability, ranging from high coupling (β-AR stimulation) to medium coupling (basal state) to low coupling (ChR-stimulation), extending further to dysrhythmic firing and dormancy. Ground-breaking recent studies in intact SAN discovered a new paradigm of SAN operation: rhythmic cardiac impulses emerge from heterogeneous local signals within and among cells of pacemaker tissue. These signals include both subthreshold LCRs and APs so that the coupled-clock theory translates into SAN tissue to operate within and among cells. How local heterogeneous signals self-organize into signaling patterns in cell clusters (or cell communities) and further synchronize to generate rhythmic cardiac impulses is currently at the frontier of our understanding.

With respect to clinical applications, failure to initiate and conduct electrical activity within the heart is associated with various cardiovascular and noncardiovascular diseases and increases as we age. Current treatment of such sinoatrial node dysfunction involves the implantation of an electronic pacemaker, which is both a costly and imperfect solution. A comprehensive understanding of SAN automaticity helps to expedite the creation of a "biological pacemaker," a superior, more physiological, and potentially less invasive treatment that would reliably and sustainably induce or reinstall cardiac pacemaker cell functions via somatic gene transfer, cell fusion, or cell transplantation [108, 109]. One type of genetically engineered biological pacemakers, based on the coupled-clock theory, activates Ca-stimulated AC1 or AC8 via virus-transfection gene therapy [48]. Indeed, efficient biological pacing has been reported in experiments with dogs in which AC1 was used as single gene therapy and in combination with HCN2 [47]. Other ideas for biological pacing include the transfer of genes encoding transcription factors [109, 110].

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