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Normal heart rhythm is initiated and regulated by an intracellular Calcium clock within pacemaker cells

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Abstract

For almost half a century it has been thought that the heart rhythm originates on the surface membrane of the cardiac pacemaker cells and is driven by voltage-gated ion channels (membrane clocks). Data from several recent studies, however, conclusively show that the rhythm is initiated, sustained, and regulated by oscillatory Ca^{2+} releases (Ca^{2+} clock) from the sarcoplasmic reticulum, a major Ca^{2+} store within sinoatrial node cells, the primary heart's pacemakers. Activation of the local oscillatory Ca^{2+} releases is independent of membrane depolarization and driven by a high level of basal state phosphorylation of Ca^{2+} cycling proteins. The releases produce Ca^{2+} wavelets under the cell surface membrane during the later phase of diastolic depolarization and activate the forward mode of $\text{Na}^+/\text{Ca}^{2+}$ exchanger resulting in inward membrane current, which ignites an action potential. Phosphorylation-dependent gradation of speed at which Ca^{2+} clock cycles is the essential regulatory mechanism of normal pacemaker rate and rhythm. The robust regulation of pacemaker function is insured by tight integration of Ca^{2+} and membrane clocks: the action potential shape and ion fluxes are tuned by membrane clocks to sustain operation of the Ca^{2+} clock which produces timely and powerful ignition of the membrane clocks to effect action potentials.

MeSH keywords

Heart Rate; Sinoatrial Node; Calcium; Sarcoplasmic Reticulum; Ryanodine Receptor Calcium Release Channel; Ion Channels

1. Introduction

The current dogma of the initiation and regulation of rhythmic heart beats has been evolved from the intertwining of theory and experimentation for almost 200 years [1,2]. Shortly after the development of quantitative membrane excitation theory by Hodgkin and Huxley in 1952 [3], the mechanism of the cardiac pacemaker initiation has been thought largely to result from interplay of voltage-gated ion membrane currents [4]. Since that time pacemaker cell research has largely focused on these essentially membrane-delimited pacemaker mechanisms. Although the ensemble of voltage and time-dependent rhythms of surface membrane ion channels ("Membrane clocks", Fig. 1A), represents the immediate cause for excitation and indeed execution of action potentials (AP) in sinoatrial nodal cells (SANC), it does not necessarily follow that this ion channel ensemble is the formal, i.e., initiating, cause of spontaneous, rhythmic APs.

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Recent, robust experimental evidence, based on confocal cell imaging, and supported by numerical modeling, puts forth a novel concept to explain normal, firing rhythm in SANC: intracellular Ca^{2+} cycling (“ Ca^{2+} clock”, Fig. 1A) ignites membrane clocks, effecting rhythmic APs. The SANC’s Ca^{2+} clock is manifested by spontaneous, but precisely timed, rhythmic, submembrane, local Ca^{2+} releases from sarcoplasmic reticulum (SR) that appear shortly before firing of the next AP (Fig. 1B–D). These Ca^{2+} releases rhythmically activate $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) inward current, prompting the membrane clocks to generate those rhythmic APs (Fig. 1B). The occurrence and rhythmicity of local Ca^{2+} releases are tightly controlled by a high degree of basal and reserve protein kinase A (PKA)-dependent protein phosphorylation [5]. Phosphorylation-dependent gradation of the Ca^{2+} clock, by regulation of the speed of Ca^{2+} cycling, is the essential regulatory mechanism of normal pacemaker rate and rhythm. Although membrane clocks do not initiate pacemaker AP, they tune the AP shape and ion fluxes to sustain function of the Ca^{2+} clock. Thus, the intracellular Ca^{2+} clock does not merely fine tune SANC AP firing, but is, in fact, the formal cause of both the basal and reserve pacemaker rate rhythms, insuring pacemaker robustness, and responsiveness by rhythmically integrating multiple Ca^{2+} -dependent functions, including those of membrane clocks and ion transporters. We believe that the new concept of the heartbeat origin replaces the current dogma of cardiac pacemaker mechanism, and thus releases the field from an “intellectual phase lock” of the membrane-delimited approach that has been perpetuated since 1960. Perhaps, the most significant implication of the new findings is that two separate field of cardiac research of how fast and strong the heart beats, are now merged within a novel, general, unifying theory [2,6]: intracellular Ca^{2+} cycling initiates the heartbeat in cardiac pacemaker cells, and, in ventricular myocytes, executes the heartbeat.

2. Spontaneous local Ca^{2+} releases in SANC, the primary heart’s pacemaker

In rabbit SANC, fluorescence imaging over the last decade has documented the occurrence of an AP-induced Ca^{2+} transient, and, importantly, in the absence of Ca^{2+} overload, confocal imaging has permitted detection of the occurrence of localized, Ca^{2+} releases (LCRs) during normal cell function [7,8]. During spontaneous beating, LCRs occur during late DD in the form of multiple locally propagating wavelets beneath the cell surface membrane (Fig. 1C,D) [7], 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[9]. While LCRs occur during the spontaneous diastolic depolarization (DD) in spontaneously firing rabbit SANC, they do not require triggering by the depolarization: spontaneous LCRs in SANC occur in the absence of membrane potential changes, i.e., during voltage clamp (“Control” in Fig. 2C and Fig. 3C), or following “chemical skinning” of the surface membrane with the detergent saponin (“Control” in Fig. 2Aa and Fig. 3A) [5,10]. Of note, however, in cat, latent atrial pacemaker cells, T-type Ca^{2+} current, activated during DD, is thought to trigger LCRs, as these are blocked by 50 μM Ni^{2+} in concentrations that inhibit T-type Ca^{2+} channels [8]; in contrast, in primary rabbit SANC, Ni^{2+} at these concentrations does not inhibit LCRs (Fig. 4)[11].

3. Spontaneous LCRs are rhythmic: The SR is a “ Ca^{2+} clock”

In the absence of regularly occurring APs, LCRs occurrences are roughly periodic and rhythmic in SANC (see Fourier spectrum of LCR activity in voltage-clamped cells in Fig. 2E “Control”). Thus, the SR can be considered as a Ca^{2+} clock. During spontaneous SANC firing, i.e. in the presence of rhythmically occurring APs, the LCR period or the speed at which Ca^{2+} Clock ticks, is reflected in the delay between the prior AP-induced, L-type Ca^{2+} channel-triggered, relatively synchronized cytosolic Ca^{2+} transient and LCR emergence later in diastole (Fig. 1D, “LCR period”). The Ca^{2+} clock’s period is, thus, slightly shorter than the cycle length (Fig. 1B); it approximately coincides with the LCR period measured during the first “would-be” cycle period after switch from spontaneous beating to voltage clamp or the period of Ca^{2+}

oscillations under voltage clamp [10]. A key point to note is that the LCR period is not fixed, but rather depends upon the speed at which SR Ca^{2+} cycling mechanisms have restituted following the Ca^{2+} release and SR Ca^{2+} depletion, and RyR inactivation effected by the prior AP, similar to what has been observed in ventricular cells [12]. Neither the SR Ca^{2+} load, nor Ca^{2+} cycling restitution status is fixed, but each changes in response to variations in 1) rate or pattern of APs, which determine net cell Ca^{2+} balance, 2) the velocity and extent of SR Ca^{2+} pumping into the SR, and 3) activation status of SR release mechanisms. Recent studies have shown that the speed at which the SR Ca^{2+} Clock “ticks” is under tight regulation by the degree of PKA-dependent phosphorylation [5]. In skinned or voltage clamped rabbit SANC, inhibition of PKA-dependent phosphorylation by PKI, a specific peptide PKA inhibitor, slows the Ca^{2+} clock speed (LCRs in Fig. 2). This indicates a crucial role of intracellular PKA-dependent phosphorylation of SR Ca^{2+} cycling proteins in the control of the Ca^{2+} clock speed, thus permitting the generation of LCRs in SANC in the basal state. Direct elevation of cAMP, or stimulation of β ARs, increases the Ca^{2+} clock speed (i.e. reduces the LCR period)(Fig. 3).

4. High levels of basal cAMP and PKA-dependent phosphorylation drive the “high speed” SANC Ca^{2+} clock in the basal state

While, basal, diastolic Ca^{2+} levels in rabbit SANC and ventricular cells do not differ, [10,13, 14] the SR Ca^{2+} clock within spontaneously firing SANC, in the basal state, restitutes and generates LCRs with an LCR period (<0.5 s), which is substantially less than in ventricular myocytes under basal conditions (~ 10 s [15]) thus allowing for LCRs to emerge sooner (following prior AP) in SANC than in ventricular cells. A recent discovery provides the clue as to why the basal SANC SR Ca^{2+} Clock speed is so rapid: high basal levels of cAMP and cAMP-dependent phosphorylation are present within SANC, i.e. even in the absence of β -AR stimulation (Fig. 5).

The high levels of basal cAMP are due to high constitutive adenylyl cyclase activity that is not driven by constitutive β -AR activation [5]. Preliminary evidence indicates that two adenylyl cyclase isoforms, Type 2 and Type 8, a Ca^{2+} -activated isoform highly expressed in the brain, are expressed in SANC, but not in other cardiomyocytes [16]. Interestingly, basal phosphodiesterase activity also appears to be elevated in SANC [17], likely as a mechanism to keep basal cAMP levels in check. Among proteins highly phosphorylated in the basal state are phospholamban (Fig. 5) and RyRs [5]; and indirect evidence suggests that L-type Ca^{2+} channels are likely to be as well [18]. 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 the Ca^{2+} release threshold (ryanodine receptors). The net effects of this phosphorylation create conditions that are required for LCR spontaneous activation during the DD in the basal state.

5. The rhythmic intracellular Ca^{2+} clock entrains normal automaticity in SANC via activation of inward NCX current

Recent experimental data, supported by numerical modeling in spontaneously firing, rabbit SANC [5,7,10,11,14,19,20] conclusively show that, LCRs emerging during the later part of DD, ignite NCX inward current (Fig. 6 and schematically illustrated in Fig. 1A–B). This imparts an exponential increase to the later part of DD [7,20] (“Nonlinear DD” in Figs 1B and 6A), prompting activation of surface membrane, voltage-gated, L-type Ca^{2+} channels to initiate an AP. An analysis of the detailed fine structure of the exponentially rising DD in SANC (Fig. 7) reveals noticeable beat-to-beat membrane potential fluctuations (Fig. 7C–D) that coincide with the occurrence of LCRs (Fig. 7E). These fluctuations reflect activation of local NCX currents by individual LCRs resulting from a stochastic nature of RyR openings [20,21].

Blocking LCRs with ryanodine (Fig. 6A–B) or blocking NCX by brief, rapid Na^+ -free superfusion (Fig. 6C–D) in rabbit SANC reduces the LCR-initiated inward current and markedly slows or abolishes spontaneous SANC AP firing. In voltage-clamped SANC, persistent, rhythmic LCRs generate rhythmic current fluctuations with the same periodicity (Fig. 2 and 3); both periodic LCRs and current fluctuations are abolished by ryanodine [22]. Both the DD variance and the amplitude of the late, nonlinear DD component are closely correlated with the changes in beating rate produced by a variety of chronotropic interventions known to influence period and/or amplitude of LCRs (e.g. ryanodine, BAPTA, nifedipine or isoproterenol)(Fig. 7F).

Entrainment of automaticity in SANC by rhythmic $\text{LCR}/I_{\text{NCX}}$ signals originating from an internal Ca^{2+} clock is not really surprising, since it has been known for long time from both experimental and theoretical studies that generation of APs in SANC can be easily entrained by rhythmic electric current pulses, if these pulses are applied in the “right” phase of the pacemaker cycle (so called “1:1 entrainment zone” in the phase response curves) [23,24]. A 2:1 entrainment pattern and other complex Wenckebach-like patterns (e.g., 5:4, 4:3, and 3:2) were also observed when pulses were applied out of the 1:1 zone. Although depolarization pulses from $\text{LCR}/I_{\text{NCX}}$ signals are generated from within the cell by the SR Ca^{2+} clock, their electrogenic influence on the excitable SANC membrane is obviously identical to that applied by an experimenter in a current-clamp configuration. Interestingly, in rabbit SANC LCRs predominantly occur within a time window of ~20% of the cycle length before an excitation [10,14], i.e., exactly where “1:1 entrainment zone” was originally discovered [23]. Also, arrhythmic beating showing various alternation patterns can be observed in rabbit SANC when the SR clock is slowed by chelation of intracellular Ca^{2+} [25], by disabling Ca^{2+} release by ryanodine [7] or by inhibition of PKA signaling [5,26].

6. Phosphorylation-dependent gradation of Ca^{2+} clock speed is the essential regulatory mechanism of normal pacemaker rate and rhythm (Fig. 8)

6.1. Slowing of the Ca^{2+} clock by PKA-inhibition

Graded reduction of PKA-dependent phosphorylation prolongs the LCR period or abolishes LCRs in permeabilized, voltage-clamped, or spontaneously beating SANC (Fig. 2, Fig. 9A–C). In spontaneously beating cells this produces parallel effects on the spontaneous AP cycle length of SANC (Fig. 8B, “Phosphorylation”). The dose response of the suppression of the spontaneous beating rate, due to the inhibition of PKA phosphorylation, closely parallels that of the suppression of phospholamban phosphorylation at serine 16 (Fig. 10B). This effect of de-phosphorylation also likely involves graded effects on L-type Ca^{2+} channels [18] to reduce Ca^{2+} influx and cytosolic Ca^{2+} , and on SR Ca^{2+} cycling proteins to decelerate Ca^{2+} cycling. In slowing the basal SR Ca^{2+} Clock and prolonging the LCR period (Fig. 9A–C), submaximal basal PKA inhibition concomitantly slows the spontaneous AP firing rate (Fig. 10). The extent to which the LCR period in individual, isolated SANC becomes increased by PKA inhibition is highly correlated with the concomitant prolongation of the spontaneous AP cycle length (Fig. 10). The time-dependent effects on beating rate, of submaximal, basal PKA inhibition and its reversal in individual SANC, are also highly correlated with the concomitant time-dependent changes effected in LCR period [5,26]. After a prolonged time of submaximal PKA inhibition or following higher levels of PKA inhibition, LCRs are no longer detectable, and the rate of spontaneous AP's markedly slows, becomes highly irregular and often ceases [5].

6.2. Acceleration of the Ca^{2+} clock by PKA-activation

As noted in above, increasing PKA-dependent phosphorylation reduces the LCR period (i.e. speeds up the Ca^{2+} clock) in permeabilized, voltage-clamped, or spontaneously beating SANC (Fig. 3; Fig. 9A, D–E). In spontaneously beating cells, the extent to which this occurs is highly

correlated with the concomitant increase in the spontaneous beating rate (Fig. 10C,D). The crucial role of RyR-initiated LCRs in mediating the chronotropic response to cAMP-dependent signaling by β -AR stimulation has been demonstrated by employing different chronotropic perturbations in the presence and absence of ryanodine. In vitro, in single SANC, the effects of the cell-permeable cAMP [16], or β -AR stimulation [11], and in the intact organism, in vivo [16], to increase the spontaneous AP firing rate or heart rate are markedly blunted in the presence of ryanodine.

6.3. The set point of physiological regulation of pacemaker rate and rhythm is determined by PKA-dependent regulation of the Ca^{2+} clock

The relative change in AP firing rate among cells and the relative LCR period prolongation by PKI, or relative LCR period reduction by β -AR stimulation are extremely highly correlated, ($r^2 = 0.97$ and this relationship lies on the line of identity, Fig. 10D). Since the Ca^{2+} clock controls the chronotropic state of the SANC via NCX (previous section), this phosphorylation-dependent gradation of the speed at which the Ca^{2+} clock ticks is the essence of both basal rate regulation and regulation of chronotropic reserve of SANC by neuromodulators [16]. This data (Fig. 10) correlating the relative beating rate increase or decrease and relative changes in PKA activation or inhibition, respectively, reveals that the basal SANC pacemaker clock speed is set to result in basal beating rate that is close to mid-point of the rate regulation range of spontaneously beating rates observed in isolated rabbit SANC (from approximately -30% to $+20\%$, i.e. it is slightly shifted towards higher rates). 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.

7. Membrane ion channels are necessary but not sufficient to sustain normal pacemaker function and regulation

7.1. Membrane ion channels and transporters regulate the intracellular Ca^{2+} clock

Robust regulation of SANC pacemaker function requires tight integration of membrane and intracellular Ca^{2+} cycling. The Ca^{2+} cycling proteins of the SR, and sarcolemmal ion channels, mutually entrain and thus tightly control each other during spontaneous basal and β -AR-stimulated SANC firing. Multiple control points are mediated by the functional dependencies of the involved proteins on Ca^{2+} , cAMP, phosphorylation, and membrane potential (Fig. 8A). The key integration elements include the L-type Ca^{2+} channels and NCX as their functions are both voltage and Ca^{2+} dependent (Fig. 1A). I_{CaL} -related Ca^{2+} influx triggers global Ca^{2+} transient that causes a global SR Ca^{2+} depletion. This resets local SR Ca^{2+} clocks within the cell (Fig. 1B, see “ Ca^{2+} clock phases: reset”) and thus synchronizes the appearance of their LCRs later in diastole. I_{CaL} is finely tuned by the Ca^{2+} transient that it triggers (via its Ca^{2+} dependent inactivation and CaMKII dependence [25]), and NCX operation is tuned by the membrane voltage during the DD. Ca^{2+} influx via I_{CaL} (and, possibly, via reverse NCX function) also “refuels” the Ca^{2+} clock by supplying Ca^{2+} to be pumped into the SR. The basal, sustained SANC Ca^{2+} clock function is supported not only by enhanced function of SR Ca^{2+} cycling proteins but also results from an enhanced L-type Ca^{2+} channel activity, as these channels seem to be highly phosphorylated in the basal state by PKA [18]. In general, modulatory changes of ion channel characteristics produced by Ca^{2+} , cAMP, and cAMP/PKA-dependent phosphorylation (see review [2] for details) can be viewed as supporting the enhanced Ca^{2+} clock operation (dotted lines in Fig. 8A), as they tune and optimize LCRs: 1) a larger Ca^{2+} influx via Ca^{2+} current enhances the SERCA Ca^{2+} pumping rate; 2) a larger I_{Kr} effects a more rapid repolarization and shorter AP duration, providing sufficient time for removal of inactivation of Ca^{2+} channels within a shorter cycle length; 3) shift in I_f activation

by high basal cAMP level and increase in the sustained current (I_{st}) limit the extent of hyperpolarization by the increased I_{Kr} , ensuring that a) LCR-activated I_{NCX} operates within the voltage range close to the I_{CaL} activation threshold and b) cell Ca^{2+} is preserved from lost that would occur via enhanced NCX function at low voltages.

7.2. While ion channels define membrane excitability, they do not initiate AP in SANC: Pacemaker cycle begins from the Ca^{2+} clock-induced acceleration of membrane depolarization during the late DD

It is important to note that while ion channels tune and support long term operation of the Ca^{2+} clock, the Ca^{2+} clock operates independently of ion channels. Under voltage clamp at the maximum diastolic potential the Ca^{2+} clock can generate LCRs for at least several cycles (Fig. 11A) until SR Ca^{2+} depletion occurs (Fig. 11B). A failure to generate the critical LCR/ I_{NCX} signals and exponentially rising DD phase leads to pacemaker failure, when Ca^{2+} influx via I_{CaL} is normal (under ryanodine or NCX blockade [7]) or even enhanced, e.g. in ischemia-like conditions [27]. Robust pacemaker function requires the activation of LCRs and I_{NCX} during the late DD [7,14,20], i.e. that crucial time when voltage-gated K^+ conductance has been stabilized and I_f has faded (due to its relatively low reversal potential), but I_{CaL} is not yet activated (Fig. 1B). Thus, the initiation of the subsequent cycle begins with LCR ignition of NCX current and DD acceleration. This deviates categorically from the present dogma about pacemaker automaticity, which envisions that pacemaker ignition begins from maximum diastolic potential. Our hypothesis relegates the events that determine the maximum diastolic potential and early DD as “after effects” of the previous AP, known as an afterpotential (i.e. after hyperpolarization in this case) that is a common feature of many cardiac cell types [1]. Earlier studies have shared our vision of initiation of true pacemaker activity that the pacemaker cycle begins with the DD acceleration. A detailed analysis of AP shapes in various regions of rabbit sinoatrial node revealed that the late **acceleration** of depolarization is observed as an earliest event within the pacemaker region [28]. Thus, intracellular Ca^{2+} cycling provides primary (i.e. depolarization-independent) rhythmic signals, which entrain sinoatrial node rhythmic excitations.

β -AR stimulation enhances I_{CaL} in the presence of ryanodine, to the same extent as in the absence of ryanodine, but the chronotropic effect of this stimulation is markedly blunted (both in vitro and in vivo) [5,11]. A minor role of I_f in cardiac pacemaker mechanism has been also previously suggested based on a variety of other considerations, including its activation kinetics, voltage-dependency, and a minor change of the basal beating rate after its blockade [10,11,19,29]. The impact of a greatly increased I_f found under ischemic-like conditions [27] fails to drive membrane potential towards the lower take-off potential (defined by the increased I_{CaL}), similarly questions a major role of this so called “the pacemaker current” [30] in the pacemaker function of SANC.

Further, graded inhibition of PKA activity with PKI reveals a wide range of reserved/basal regulation of SANC spontaneous beating rate via PKA (discussed in previous section, Figs. 8B and 10); but this does not involve I_f , because this current is cAMP-(but not PKA-) dependent [31,32]. As discussed above, β -AR stimulation of SANC beating rate is markedly blunted in the presence of ryanodine, indicating a minor role of the membrane clock (including I_f) in the rate regulation following β AR stimulation [5]. Although, Bucchi et al [33] found almost no difference in the effect of membrane-permeable cAMP (CPT-cAMP) on the SANC beating rate in the presence or absence of ryanodine, this result has been reinterpreted [5], as the increase in beating rate produced by CPT-cAMP in control conditions (Fig. 8C) was two-fold greater than reported by Bucchi et al. Further, a blockade of I_f by Cs^+ resulted in minor changes of β -AR stimulation effect [11]. Thus, in our view the SANC rate regulation 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 . Finally, while knocking out either cardiac RyR (type 2) or NCX results in embryonic lethality (review [2]), hearts of mouse embryos lacking HCN4 (i.e. I_f channel) contract rhythmically, albeit at a reduced (approx. 50%) rate, that is, “a basal heart rate can be sustained without I_f ” [34]. Thus, the portrayal of I_f as “the pacemaker current” (i.e. almost entirely mediating the cardiac pacemaker function) is no longer valid.

8. Testing control points of the Ca^{2+} clock: evidence for Ca^{2+} -driven pacemaker mechanism in SANC

Multiple lines of evidence support the novel concept that normal pacemaker function is based on functional integration of membrane and submembrane Ca^{2+} cycling (Figs. 1A,B, and 8A). Any factors or interventions that either directly or indirectly influence LCRs (i.e. their period and/or signal mass) and/or global Ca^{2+} transient (resetting LCRs) have major consequences for spontaneous beating:

1. Graded suppression or complete blockade of Ca^{2+} cycling by buffering intracellular Ca^{2+} (Fig. 8B, “Cytosolic Ca^{2+} ”) slows and blocks beating. In addition to abolishing Ca^{2+} release following excitation and abolishing LCRs, chelation of Ca^{2+} by BAPTA may inhibit Ca^{2+} -calmodulin dependent facilitation of I_{CaL} [25] and affect other Ca^{2+} -dependent components of membrane clocks.
2. Ryanodine, a specific inhibitor of RyR function, produces graded suppression or blockade of LCRs, and, in a concentration dependent manner ($\text{IC}_{50}=3 \mu\text{M}$), reduces the spontaneous SANC beating rate; at higher concentrations, ryanodine can abolish beating [7] (Fig. 8B, “Ryanodine receptors”).
3. In contrast to ryanodine, which specifically blocks the Ca^{2+} clock, a nifedipine blockade of L-type Ca^{2+} channels directly blocks the membrane clock and I_{CaL} -mediated Ca^{2+} influx. This prevents resetting and refueling (see section 7.1) the Ca^{2+} clock leading to SR Ca^{2+} depletion and LCR abolition, culminating in pacemaker failure.
4. Cyclopiazonic acid, a selective Ca^{2+} ATP-ase antagonist, interferes with SR Ca^{2+} pumping function and slows SR Ca^{2+} clock. Application of this drug significantly increases LCR period and decreases spontaneous beating rate (~ by half) of both guinea pig SANC [35] and rabbit SANC [36].
5. Under conditions simulating ischemia in rabbit SANC, pacemaker function fails when I_{CaL} and I_f increase, I_{Kr} is unchanged, and I_{CaT} and I_{NCX} decrease [27,37]. The failure of normal automaticity in this case is associated with the elimination of the late exponential phase of the DD. While I_{CaT} is activated during the late DD, this current has a minor contribution into SAN pacemaker function in relatively large mammals, such as rabbit [38]. LCRs, in contrast, have major effects on the rabbit SANC beating rate via activation of NCX and accelerating the late DD (section 5) and are reduced by myocardial ischemia [39,40].
6. Variations in the basal LCR period or the first “would-be” cycle during voltage clamp (both parameters assess the Ca^{2+} clock period) among different cells are tightly linked to variations in their spontaneous cycle length [10]. The LCRs emergence closely precedes the spontaneous APs, and thus can indeed prompt activation of the APs.
7. Cessation of spontaneous AP's by voltage clamp at the maximum diastolic potential leads to SR Ca^{2+} depletion and LCR abolition (Fig. 11). Removal of the voltage clamp is followed by the gradual recovery of the control spontaneous cycling rate, and this

gradual recovery is highly correlated with the return of LCRs and the recovery of the control LCR period [10].

8. Physiological regulation of Ca^{2+} clock by neuromediators: There is tight coupling among the degree of PKA-dependent protein phosphorylation, the speed at which the SR Clock generates LCRs, and the concomitant rate at which spontaneous AP's are generated (Figs. 2,3,8B,9,10; see section 6 for details).

9. Novel numerical pacemaker models to test Ca^{2+} clock-driven pacemaker mechanism

Initial tests of the ability of LCRs during the DD to command the AP firing rate utilized a primary rabbit SANC model developed by Zhang et al. [41] When experimentally measured, submembrane, LCR waveforms were introduced into this model, spontaneous AP firing rate was easily entrained at a rate determined by the LCR period [19].

Subsequently, Kurata et al., model [42] described Ca^{2+} concentration in sub-membrane space and predicted strong negative chronotropic effect of Ca^{2+} chelation in this critical location. A novel pacemaker model [14] included LCR in the sub-membrane space and reproduced, for the first time, the negative chronotropic effect of ryanodine, and predicted a new powerful mechanism of rate regulation by varying Ca^{2+} release rate and phase: the larger release resulted in the larger I_{NCX} that, in turn, allowed wider rate regulation range by the phase of the release covering basically the entire physiological range. In terms of the fine DD structure, the model showed that the LCR-activated NCX current imparts exponentially rising part to the late DD that culminates in an AP upstroke [14].

A subsequent upgrade of the local Ca^{2+} release model reproduced individual stochastic LCRs (a multicompartiment SR model) [5,20]. Varying frequency, size, and phase of the LCRs according to experimental data, this model predicted the wide range of chronotropic effects that were experimentally observed with graded PKA activity, that is, basal and reserve rate regulation (Fig. 10D, "Model PKI" and "Model ISO") via a stochastic ensemble of local NCX currents induced by the LCRs [5]. The model also provided further details of fine DD structure including its fluctuations and exponentially rising late DD component observed experimentally shortly before the excitation (Fig. 7, described in section 5), as well as modest beat-to-beat cycle variations due to the stochastic occurrence of the LCRs [20]. This model identified substantial benefits of interactions of the rhythmic LCRs and the membrane ion channel clock in terms of overall performance of the new pacemaker mechanism. Under conditions when ion channels operating alone in numerical models fail to generate rhythmic AP's, stable and rhythmic AP firing resumes when the timely and powerful prompt to the membrane sent by SR via LCRs and NCX current is introduced into the model [26].

The numerical model approaches described above illustrated that the concept of Ca^{2+} release-ignited excitation is indeed operational in primary SANC, based on the existing knowledge on LCRs and SANC-specific ion channels. However, the Ca^{2+} release function in these model simulations was, either substituted by the experimental waveform, or approximated by a phenomenological mathematical function (in fact, a sine function). The most recent approaches to quantitatively describe Ca^{2+} integrated SANC function has included spontaneous Ca^{2+} release mechanisms [43] and local Ca^{2+} dynamics [44]. More specifically, this approach has developed a new model [43] of primary rabbit SANC, in which an integrated Ca^{2+} release mechanism is controlled by the SR Ca^{2+} load (modified from Shannon et al. 2004 [45]). This more mechanistic model now predicts SR Ca^{2+} depletion-refilling dynamics measured by Fluo-5N [36,43] in spontaneously beating SANC and exhibits spontaneous Ca^{2+} oscillations under voltage clamp. Varying the Ca^{2+} clock model parameters results in a graded control of the timing of spontaneous Ca^{2+} release and the pacemaker rate. This result was corroborated

by the experimental finding that cyclopiazonic acid, an inhibitor of SR Ca^{2+} ATPase, effects an ~50% reduction of the spontaneous beating rate of rabbit SANC [36]. These recent numerical model studies thus show that: 1) periodic SR Ca^{2+} refilling and release indeed define (quantitatively rather than speculatively or phenomenologically) the Ca^{2+} clock within SANC; and 2) beat-to-beat interaction of this Ca^{2+} clock with the membrane clocks results in a robust pacemaker function with an effectively controlled beating rate.

The great acceleration of computational power of modern computers permits development of an extension of the presently available models to simulate local Ca^{2+} dynamics in the submembrane space of SANC, based on approximation of Ca^{2+} diffusion and stochastic activation of RyR clusters (Ca^{2+} release units) [44]. These simulations predict the occurrence of local stochastic Ca^{2+} wavelets with spatiotemporal properties similar to those found for LCRs in SANC. In perspective, when the above two modeling approaches converge, a rather sophisticated, powerful SANC model integrating function of membrane channels, SR Ca^{2+} release mechanism, and local Ca^{2+} dynamics will likely emerge.

SUMMARY

The true cardiac pacemaker mechanism is complex and includes a balanced integration of both intracellular and membrane delimited processes. While the SANC ion channel membrane clocks (Fig. 1A) are necessary to effect an AP, they are not sufficient in our opinion to ignite an AP, or to generate rhythmic APs at normal rates. Rather, both experimental data and numerical simulations reviewed here demonstrate that an intracellular Ca^{2+} clock (Fig. 1A) and the rhythmic LCRs it generates do not merely “fine tune” the normal automaticity of SANC, but by igniting excitations from within the cell (see “Ignition” in Fig. 1A), represent the formal cause of APs. Thus, the rate at which the Ca^{2+} clock ticks, controls rhythmic, spontaneous AP firing across the broad range of physiologic firing rates. In short, variations of internal Ca^{2+} cycling within SANC result in corresponding variations in LCRs that are tightly linked to gradations in their normal automaticity. The spontaneous, but tightly controlled, rhythmic, SR Ca^{2+} clock of SANC insures the stability of the basal rhythm and sets the basal firing rate by integrating multiple Ca^{2+} -dependent functions and rhythmically interacting with one, igniting the ensemble of surface membrane clocks to effect APs. Variable degrees of intrinsic PKA-dependent protein phosphorylation, and its attendant variations in intracellular Ca^{2+} regulate the time that the SR Ca^{2+} clock keeps, thus regulating the rate at which LCRs occurs, and modulate the voltage- and time-dependent kinetics of the membrane clocks, thus insuring that they produce APs with characteristics commensurate with given rhythmic ignition rates commanded by LCRs. Interfering with SR internal Ca^{2+} cycling interferes with normal pacemaker functions, just as does interfering with membrane ion channels that generate the AP upstroke and repolarization (Fig. 8B). Thus, the true initiation of cardiac pacemaker automaticity begins during the late part of the DD when the Ca^{2+} clock ignites the surface membrane excitation. Earlier events such as the maximum diastolic potential and early, slow DD ensure recovery of the membrane clocks that are activated during the prior AP and need to be reset before next AP.

In the intact organism, integrated autonomic nervous system signals confer another layer of regulation of the intrinsic cAMP/PKA/ Ca^{2+} signaling and of the SANC, SR Ca^{2+} Clock and membrane clocks of SANC: vagal stimulation of cholinergic receptors predominates in the basal state, keeping the SR Ca^{2+} clock in check; in response to a demand for higher heart rates, stimulation of cholinergic receptors wanes, and sympathetic stimulation of β -adrenergic waxes; at the maximum chronotropic rate, β -AR activation of PKA-dependent phosphorylation is maximal, and the SR Ca^{2+} clock runs at full speed, activating NCX and igniting the membrane clock at a maximum rate in order to achieve the maximum heart rate.

The evidence for the crucial role of Ca^{2+} and rhythmic spontaneous Ca^{2+} releases in the initiation and regulation of normal cardiac automaticity provides the key that reunites pacemaker and ventricular cell research [2,6]. The separate achievements of reductionist approaches to elucidate how strength of contraction of ventricular myocytes and the spontaneous beating rate of pacemaker cells are regulated now converge: Ca^{2+} cycling into and from the SR by proteins common to both ventricular myocytes and pacemaker cells can be portrayed as Ca^{2+} clock. The mutual entrainment of the Ca^{2+} clock and surface membrane ion channel clocks regulate the duty cycle of both cell types. Thus, a general theory of cardiac inotropy and chronotropy emerges.

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Figure 1.

A: Schematic representation of the idea that Ca^{2+} clock ignites ion channel membrane clocks to entrain normal automaticity in cardiac pacemaker cells (see text for details). **B:** A schematic illustration of the fine structure of the diastolic depolarization (DD) following of spontaneous action potential in rabbit SANC shown together with inward ion currents and related Ca^{2+} signals. The nonlinear, exponentially rising, late DD part is shown as a gray area between membrane potential curve and an extrapolation of the initial, linear DD part (modified from [37]). **C,D:** Confocal line scan images of Ca^{2+} signals (Fluo-3) measured in rabbit SANC with different orientation of the scanning line. Trace in panel C is simultaneous action potential recordings in perforated patch clamp configuration. Trace in panel D is the time course of the average Ca^{2+} signal along the scan line. Local Ca^{2+} Releases (shown by white arrows) occur under cell surface membrane in the later part of diastolic depolarization. Modified from [14].

Figure 2.

Suppression of cAMP-mediated, PKA-dependent signaling in SANC decreases frequency and size of LCRs in permeabilized and intact SANC. **A:** Confocal linescan images of a representative saponin-permeabilized SANC bathed in 100 nmol/L free $[Ca^{2+}]$ before (top), and after (bottom) superfusion with 15 μ M PKI, a specific peptide inhibitor of PKA catalytic subunit. **B:** The average frequency (normalized per 1 s and 100 μ m) and size of LCR in skinned SANC in control conditions (72 LCR, $n = 4$) and 15 μ M PKI (20 LCR). * $P < 0.05$. Error bars indicate standard error of mean. **C** and **D:** Simultaneous recordings of membrane potential or current (top), confocal linescan image (middle), and normalized fluo-3 fluorescence (bottom) averaged over the linescan image, in a representative spontaneously beating SANC with intact

sarcolemma before and during voltage clamp to -10 mV in control (**C**) and following PKA inhibition ($8 \mu\text{M}$ PKI) (**D**). Fast Fourier transform (FFT) of Ca^{2+} (**E**) and membrane current (**F**) fluctuations during voltage clamp in control and after PKI. Because current fluctuations during voltage clamp were imposed on the total membrane current, each data set was fit with a nonlinear regression line that was subtracted to give a difference signal to minimize frequency interference. From [5].

Figure 3. Stimulation of cAMP-mediated, PKA-dependent signaling in SANC increases internal Ca^{2+} oscillation frequency. **A:** Confocal linescan images of a representative saponin-skinned SANC bathed in 100 nM $[\text{Ca}^{2+}]$, before (top), and after (bottom) superfusion with 10 μM cAMP. **B:** Fast Fourier transform (FFT) of Ca^{2+} fluctuations of the cell in panel **A** in control conditions and during superfusion with cAMP. **C** and **D:** Simultaneous recordings of membrane potential or current (top), linescan image (middle), and normalized fluo-3 fluorescence (bottom) averaged over the linescan image, in a representative spontaneously beating cell with intact sarcolemma before and during voltage clamp to -10 mV in control (**C**) and following exposure

to a β -AR agonist (0.1 μ M isoproterenol, ISO) (**D**). **E** and **F**: FFT of Ca^{2+} (**E**) and membrane current fluctuations (**F**) during voltage clamp in control and after ISO. From [5].

Figure 4.

Local Ca^{2+} releases persist during spontaneous beating and under voltage clamp to -70 mV, when T-type Ca^{2+} current is blocked by Ni^{2+} in rabbit SANC. **A:** recordings of APs (top), line-scan image (middle) and normalized subsarcolemmal fluorescence averaged spatially over the image width in a representative SANC during superfusion with 50 μM Ni^{2+} . **B:** control recordings of line-scan image and normalized subsarcolemmal fluorescence in the same cell before exposure to Ni^{2+} . From [10].

Figure 5.

High basal level of cAMP and cAMP-mediated, PKA-dependent phospholamban (PLB) phosphorylation in SANC. **A:** Average content of cAMP in suspensions of SANC, atrial or ventricular myocytes, and changes in cAMP level in SANC following suppression of adenylyl cyclase activity with 400 μ M MDL (an adenylyl cyclase inhibitor) or β -AR stimulation (1 μ M isoproterenol, ISO). **B:** Left, Western blots of the basal level of phosphorylated at serine16 (P-PLB) and total PLB in SANC and ventricular myocytes; right, average values of phosphorylated PLB normalized to total PLB. **C:** Phosphorylated PLB and total PLB at base line and in response to graded increases in AC inhibition by MDL (note that the concentration of MDL increases from right to left). **D:** Typical Western blots of the basal level of PLB phosphorylation and that following PKA inhibition (15 μ M PKI, a specific peptide inhibitor of PKA catalytic subunit), or β -AR stimulation (1 μ M ISO). **E:** Average changes in phosphorylated PLB induced by maneuvers in (C and D) (MDL concentration was 400 μ M). * $P < 0.05$. Error bars indicate standard error of means. From [5].

Figure 6.

Local Ca^{2+} releases (LCRs) ignite rhythmic APs via activation of NCX current imparting an exponential increase to the later part of the diastolic depolarization (“nonlinear DD”) in rabbit SANC. **A:** Ryanodine abolishes the LCR-mediated AP ignition early in the cycle by inhibition of the nonlinear DD (gray area shown by arrow). Modified from [22]. **B:** Ryanodine (3 μ M, 4 min) inhibits submembrane Ca^{2+} increase (*) and inward current (#) during a simulated diastolic depolarization (bottom) without affecting peak I_{CaL} . Inset shows the indicated part of the current record at greater magnification. **C:** An inhibitory effect of a 10s Na^+ -free spritz (arrow) on inward current during the voltage ramp protocol (bottom). **D:** Linescan image of Ca^{2+} release with superimposed AP records during rapid and brief superfusion with a solution in which Na^+ was replaced by Li^+ . Note that the maneuver blocked the subsequent AP firing. The line superimposed on the last AP preceding spritz of Na^+ -free solution is a copy of the residual membrane potential oscillation observed during the Li^+ solution spritz. From [7].

Figure 7.

Membrane potential fluctuations resulting from LCRs in rabbit SANC impart an exponential phase to the late DD that controls their chronotropic state. **A:** Schematic illustration of the assessment of the non-linear DD component (NDDC). **B:** Eight successive APs with seven 90-ms recordings of DD marked. **C:** Superimposition of 7 individual DDs marked in B and their mean (bold). **D:** Residual voltage after subtracting the mean of a group records from 7 successive DDs. Note the larger beat-to-beat deviations as the DD approaches the AP upstroke. **E:** Time course of DD variance (curve) and of the relative LCR occurrences (columns) observed at different times before AP upstroke (166 measurements in 24 cells under control conditions). **F:** Changes in DD fluctuations and NDDC amplitude versus changes in beating rate with respect to chronotropic interventions. From [20].

Figure 8.

A: Schematic illustration of functional integration and regulation of membrane and submembrane Ca^{2+} cycling to control pacemaker function via NCX-mediated ignition of rhythmic APs. The thick line indicates spontaneous SR Ca^{2+} cycling (see text for details). Modified from [5]. **B:** Spontaneous beating of rabbit SANC critically depends upon Ca^{2+} -related mechanisms and protein phosphorylation. Bars show a decrease in the beating rate (% control) induced by different drugs that affect Ca^{2+} cycling [ryanodine (Ry), BAPTA-AM], NCX (Li^+ substitution for Na^+), protein phosphorylation (PKI, H-89, MDL), or ion channels: I_f (Cs^+) or T-type Ca^{2+} current (Ni^{2+}). PKI and H-89 are protein kinase A inhibitors, and MDL is an adenylyl cyclase inhibitor. From [22]. **C:** Disabling RyR markedly reduces the stimulatory

effects of CPT-cAMP on the spontaneous beating rate of rabbit SANC. Left panel: typical examples of the change in the spontaneous beating rate of rabbit SANC in response to CTP-cAMP, before and after exposure to ryanodine; right panel: the average effects; error bars indicate standard error of mean, * $P < 0.05$. From [5].

Figure 9.

Suppression (PKI, a specific inhibitor of PKA, 14–22 amide) or stimulation (isoproterenol, ISO) of PKA-dependent phosphorylation have opposite effects on frequency and spatio-temporal properties of LCRs in intact, spontaneously firing rabbit SANC. **A:** Confocal linescan images of a representative nodal cells depicting AP-induced Ca^{2+} transients and LCRs during spontaneous beating in control and when PKA phosphorylation was inhibited by PKI or stimulated by ISO. **B** and **C:** Histograms of LCR period and size (full width at half maximum) in control ($n = 4$ cells, 58 LCRs) and after superfusion with 5 μM PKI ($n=4$ cells, 25 LCRs). **D** and **E:** Histograms of LCR period and size in control (8 cells, 42 LCRs) and after superfusion with 0.1 μM ISO (8 cells, 89 LCRs). From [5]

Figure 10.

A: The PKI effect to increase the spontaneous cycle length is linked to its effect on the LCR period. Note that this relationship lies above the line of identity (dashed line), indicating that the period of LCRs is shorter than the spontaneous cycle length. **B:** Typical example of APs recorded in a rabbit SANC before, during superfusion with 15 μM PKI, and during washout of the drug. The relationship of PKI-induced suppression of SANC beating rate (solid line) and PLB phosphorylation of SANC suspensions (dashed line). Inset shows Western blots of phosphorylated PLB and total PLB in response to increasing PKI concentrations. Error bars indicate standard error of mean. **C:** Relationship between LCR period and spontaneous cycle length is shifted to shorter periods by β -AR stimulation with 0.1 μM ISO. Dashed line is the line of identity. **D:** The relative PKI and ISO effects to alter spontaneous cycle length over a wide range are linked to their effects on the LCR period within the same cells. Note that this relationship conforms to the line of identity. Square symbols and solid line depict the experimentally obtained data; triangular symbols depict the average data simulated by numerical model using experimentally measured changes in LCR characteristics and phase. Modified from [5].

Figure 11.

A: LCR occurrences in the absence of changes in membrane potential in SANC with intact sarcolemma. Recordings of membrane potential (i), linescan image (ii), and normalized subsarcolemmal fluorescence (iii) averaged spatially over the band indicated by double headed arrow in (ii). (iv) Average total signal mass of LCRs during spontaneous beating and during voltage clamp ($n = 14$). **B:** Voltage clamp (VC) at the maximum diastolic potential decreases SR Ca^{2+} content and intracellular $[\text{Ca}^{2+}]$ in rabbit SANC. Spontaneous Ca^{2+} transients and caffeine-induced Ca^{2+} releases, indexed by F/F_0 , in a representative cell during spontaneous beating (i) and in a representative cell after several seconds of voltage clamp (iii). (ii) Average response to caffeine normalized to Ca^{2+} transient in cells during spontaneous beating and cells subjected to several seconds of voltage clamp. (iv) Averages of submembrane diastolic $[\text{Ca}^{2+}]$ before, during, and after voltage clamp in nine cells. * $P < 0.05$. Error bars indicate standard error of mean. From [10].