Review

The Emergence of a General Theory of the Initiation and Strength of the Heartbeat

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Abstract. Sarcoplasmic reticulum (SR) Ca^{2+} cycling, that is, the Ca^{2+} clock, entrained by externally delivered action potentials has been a major focus in ventricular myocyte research for the past 5 decades. In contrast, the focus of pacemaker cell research has largely been limited to membrane-delimited pacemaker mechanisms (membrane clock) driven by ion channels, as the immediate cause for excitation. Recent robust experimental evidence, based on confocal cell imaging, and supported by numerical modeling suggests a novel concept: the normal rhythmic heart beat is governed by the tight integration of both intracellular Ca^{2+} and membrane clocks. In pacemaker cells the intracellular Ca^{2+} clock is manifested by spontaneous, rhythmic submembrane local Ca^{2+} releases from SR, which are tightly controlled by a high degree of basal and reserve PKA-dependent protein phosphorylation. The Ca^{2+} releases rhythmically activate $\text{Na}^+/\text{Ca}^{2+}$ exchange inward currents that ignite action potentials, whose shape and ion fluxes are tuned by the membrane clock which, in turn, sustains operation of the intracellular Ca^{2+} clock. The idea that spontaneous SR Ca^{2+} releases initiate and regulate normal automaticity provides the key that reunites pacemaker and ventricular cell research, thus evolving a general theory of the initiation and strength of the heartbeat.

Keywords: cardiac excitation, contractility, pacemaker mechanism, calcium cycling, heart rate regulation

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1. Introduction

Heart performance, in its most evident form, is gauged by how often the heart beats and the strength of each beat, that is, cardiac chronotropy and inotropy, respectively. In the basal state, only a fraction of the maximum cardiac performance is utilized, but during stress, for example, dynamic exercise, the ability of the heart to acutely beat faster and stronger when the demand for cardiac output increases is central to the vertebrate survival instinct. Different types of cells within the heart, that is, pacemaker cells within the sinoatrial node (SAN) and ventricular myocytes, determine how fast and strong, respectively, the heart beats.

The heart's pacemaker cells normally generate spontaneous rhythmic changes of their membrane potential, thereby producing roughly periodic spontaneous action potentials (AP's), that is, normal automaticity. Pacemaker cells within the heart having "Clocks" with the briefest rhythmic periods "capture" or trigger other excitable cells. SAN pacemaker cells (SANC) are the dominant cardiac pacemaker cells, because they exhibit shorter periods between spontaneous AP's than do atrioventricular nodal cells or His-Purkinje cells. Thus, SANC initiate the cardiac impulses by generating spontaneous AP's that are conducted to the ventricle and capture ventricular cells by initiating AP's in these cells and entrain their duty cycle.

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. Early studies of the origin of the heart's function focused simultaneously on combined heart properties, that is, on both excitability and contraction (Fig. 1A). However, with development of quantitative membrane excitability theory in 1950s, research into mechanisms of pacemaker function and contraction have proceeded along separate paths (Fig. 1: B – J and L – S), often in isolation of each other. Research on the duty cycle of ventricular myocytes, that is, contraction, has been dominated by studies of intracellular Ca^{2+} regulation, that is, by proteins that regulate Ca^{2+} flux into and out of the cytosol (Ca^{2+} cycling), particularly those of sarcoplasmic reticulum (SR), the major intracellular Ca^{2+} store in the cells. In contrast to ventricular myocytes, the field of pacemaker research became dominated by the idea that pacemaker function could solely be accounted for by the ensemble of sarcolemmal ion channels whose ion currents reciprocally activate each other to produce spontaneous AP's.

Advances in genomics, proteomics, immunohistochemistry, and confocal imaging have and continue to provide crucial information regarding expression, local-

ization, and functions of proteins within these two cardiac cell types. While recognition of the importance of Ca^{2+} cycling in pacemaker function has been late to emerge, recent evidence that rhythmic spontaneous Ca^{2+} releases from SR initiate and regulate normal automaticity provides the key that reunites pacemaker and ventricular cell research (Fig. 1: K and T). The separate achievements of reductionist approaches to elucidate how the ventricular myocytes regulate their strength of contraction via variations in Ca^{2+} cycling and how the spontaneous beating rate of pacemaker cells is regulated converge: Regulation of sequestration and release of Ca^{2+} to and from internal stores by proteins common to both ventricular myocytes and pacemaker cells is therefore a general regulatory mechanism of the duty cycle of both cell types, that is, contraction and rhythmic excitation, respectively.

2. Early chemical and electrical concepts of the initiation of muscle function: Galvani to Bernstein

The idea that the heart is driven by electricity was raised just a little over 200 years ago when Luigi Galvani discovered that electrical stimulation of a frog's heart lead to cardiac muscle contraction. Further refinement of instrumentation (mainly galvanometers and electrodes) allowed recording electrical currents from muscle and nerve (1, 2), and from the heart itself, that is, electrocardiogram (3). The principles of how electrical signals relate to the muscle action were first formulated by DuBois-Reymond based upon the novel concept of electromagnetism of Ampere (in 1820) and Faraday (in 1831). In 1850, Helmholtz established that the speed of electrical transmission in nerves is substantially less than that in conductors, suggesting an alternative, chemical-molecular nature for muscle action. Based on the analysis of molecular composition of muscles (including glycogen and myosin), a pure chemical theory of muscle action was proposed by Herman. His idea was that the stimulated region undergoes a chemical alteration (1867–1870, "alteration" theory) accompanied by a cyclical energy release (4). Hermann considered all recordings of electricity within living tissues as artifacts of more fundamental physiological processes. The electrochemical theory of muscle action was suggested by Jules Bernstein in 1871, who conceptualized that muscle fibers consist of a protein core, binding molecules of different polarity in a very specific spatially organized manner.

Fig. 1. Emergence of a general theory of the initiation and strength of the heartbeat. Research on membrane excitation and contractile function proceeded along separate paths (separated by a dashed line) until the critical importance of Ca^{2+} cycling became evident not only in cardiac muscle (ventricular or atrial cells), but also in normal pacemaker cells (see text for details).

3. Excitation-contraction coupling: the duty cycle of skeletal and cardiac muscle cells

In 1868 Bernstein plotted a galvanometer response to nerve excitation that was, in fact, the first record of an “action potential” (5). Three years later, he discovered that in muscle, an AP occurs before the onset of contraction (6), suggesting a fundamental paradigm of the muscle duty cycle as electric excitation (E) initiating contraction (C), or simply, EC-coupling in current

terminology.

3.1. Development of membrane excitability theory and initial studies of muscle excitation

In 1902 Bernstein abandoned his initial speculative electrochemical theory. By 1912, based on work of Ostwald and Nernst (the Nernst’s Equation derived in 1889), he developed and experimentally proved the membrane theory of bioelectric potentials (4, 7, 8). This new theory linked the bioelectric potential of nerve

and muscle to ion gradients across their cell membrane. At the same time, he also suggested that a mechanism of nerve/muscle excitability was a sudden reduction of the K^+ permeability of the membrane, which reduced the membrane potential to zero.

In 1949, testing the effect of Na^+ removal on membrane potentials in large diameter squid neurons, Hodgkin and Katz (9) concluded (contrary to the original Bernstein K^+ -driven excitability theory) that Na^+ was responsible for the AP upstroke and positive membrane polarization. In the same year, Ling and Gerard invented the glass microelectrode that allowed the activity from individual cells to be recorded, and Kenneth Cole suggested placing a second electrode inside the cell in order to inject the current, and to voltage clamp the interior of the cell (10). In 1952 Hodgkin and Huxley applied the voltage clamp approach to nerve cells of a giant squid and established current-voltage relationships for Na^+ and K^+ currents (11). Based on these data, they suggested a new quantitative theory of membrane excitation, that is, of the AP generation (Fig. 1B). According to their theory, the movement of selective ions through the excitable membrane was determined by separate voltage-dependent “gates” with simple kinetics described by differential equations.

Using the new microelectrode technique, Weidmann, initially together with Coraboeuf (12), greatly advanced the knowledge about cardiac muscle electrophysiology. Weidmann confirmed the application of ionic theory to the cardiac muscle and explained the differences between cardiac AP's and those of nerve and skeletal muscle. His findings include the genesis of the resting membrane potential, the increased permeability to Na^+ during membrane depolarization, high membrane resistance during the AP plateau, and the definition of threshold potential (10, 13).

3.2 The key role of Ca^{2+} in EC-coupling mechanism

In skeletal muscle, the AP, as originally demonstrated in nerve, largely reflects the ionic flux via Na^+ and K^+ channels on the cell's surface membrane (14). It was inferred from early studies of contractile mechanics and energetics of skeletal muscle that the AP initiated an “active state” within muscle cells that resulted in contraction (15) (Fig. 1L). The aforementioned discoveries of mechanism of EC-coupling in skeletal muscle were soon translated to cardiac muscle (16, 17). Because studies in isolated myofilament preparations showed that Ca^{2+} caused myofilament interaction, it was further inferred that in intact muscle cells, the active state included a transient rise in cytosolic $[\text{Ca}^{2+}]$ that coupled E to C (18). In fact, heart research had been

“tuned in” to the crucial importance of extracellular Ca^{2+} in the cardiac muscle duty cycle since 1883, when Ringer's London tap water mistake illuminated the field (19). Subsequent studies identified the SR and its Ca^{2+} pump, and the idea that myofilament relaxation occurs following pumping of Ca^{2+} back into the SR was born. It was discovered that Ca^{2+} pumping into the SR is accomplished mainly by the SR Ca^{2+} ATPase or SERCA. The Ca^{2+} pump isoform in the heart, SERCA2A, is the dominant mechanism to remove Ca^{2+} from the cytosol following excitation/contraction, but its relative contribution varies among species. In most mammalian ventricular myocytes (including rabbit, guinea pig, ferret, dog, cat, and human), SERCA function accounts for about 60%–75% of Ca^{2+} extrusion, with 25%–40% attributable to the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (20).

Specific differences between heart and muscle cells became evident: in skeletal muscle, Ca^{2+} release from the SR was initially thought to be exclusively voltage-dependent, that is, initiated mainly by surface membrane depolarization; in cardiac muscle, Ca^{2+} release was linked to Ca^{2+} influx during an AP. Cardiac muscle cells produced a much broader AP than in skeletal muscle, due to a slow inward current [discovered by Reuter in 1966 (21) and later named L-type Ca^{2+} current (I_{CaL})], that caused Ca^{2+} influx during the AP. Early studies (22) had demonstrated a “memory” within heart muscle that produces contractions of differing amplitudes in response to changes in the rate or pattern of stimulation. It was later inferred that staircases in contraction amplitudes prior to achieving a new steady state involved changes in Ca^{2+} loading and release from the SR (23), which occur over several beats, due to beat-dependent net changes in cell Ca^{2+} . It was later discovered, in mechanically skinned cardiac cell fragments, that application of graded $[\text{Ca}^{2+}]$ triggers graded Ca^{2+} releases from the SR (24, 25), a process referred as Ca^{2+} -induced Ca^{2+} -release (CICR) to generate graded transient increases in cytosolic Ca^{2+} that initiate graded contractions. It was shown that ryanodine, an alkaloid that makes SR Ca^{2+} release channel leaky and inefficient, greatly altered the contractile state of mammalian ventricular myocardium (26, 27). This extremely important pharmacological tool was used in numerous later studies to isolate, purify, and clone the ryanodine receptor (RyR), permitting researchers to assess the contribution of Ca^{2+} release in the function of different cardiac cell types.

The advent of intracellular Ca^{2+} indicators permitted direct demonstrations that the AP does indeed result in a release of Ca^{2+} from the SR, resulting in a transient increase in cytosolic Ca^{2+} that activates the myofila-

ments to drive contraction. Cytosolic Ca^{2+} transients evoked by AP's were demonstrated in 1978 by Allen and Blinks (28) in atrial muscle and in 1980 by Wier (29) in Purkinje fibers by using aequorin, a Ca^{2+} -binding photoprotein. Two distinct Ca^{2+} signals were indeed identified: one from Ca^{2+} entry through the surface membrane and another from SR Ca^{2+} release, the latter being abolished by ryanodone in Purkinje fibers (30) and ventricular muscle (31). Thus, the earlier inference that Ca^{2+} releases triggered by an AP couples E to C was correct, and the concept of "excitation-induced- Ca^{2+} -release" evolved (Fig. 1P).

It was also discovered that increases in Ca^{2+} beneath the cell surface membrane and the mechanical strain during contraction cause feedback on ion channels and transporters underlying the AP (32). One crucial aspect of such tuning of excitation by Ca^{2+} release in ventricular myocytes occurs during the AP and involves Ca^{2+} removal from the cell to balance the Ca^{2+} influx via L-type Ca^{2+} channels. This is achieved, in part, by the cell surface membrane NCX, which extrudes one Ca^{2+} for three Na^+ , a process that generates an inward current (I_{NCX}) that affects the shape of the repolarization phase of the AP (33), and the diastolic membrane potential as well.

Subsequent research on the cardiac muscle duty cycle was dominated by the studies of the AP-induced local Ca^{2+} release mechanisms. Detailed biophysical studies showed that CICR from the SR is initiated by Ca^{2+} influx via L-type Ca^{2+} channels. The graded I_{CaL} activated by various membrane depolarizations resulted in respective graded Ca^{2+} releases, the relationship referred to as "gain" of EC-coupling, characterizing the ultimate performance of the entire EC-coupling machinery. The gradeness of CICR is under firm local control (34) by local Ca^{2+} fluxes of individual L-type Ca^{2+} channels in a tight 12-nm "dyadic space" separating the surface channels and RyRs, Ca^{2+} release channels in junctional SR. The most recent achievements, including "nanoscopic" details of Ca^{2+} channel-RyR crosstalk, and fundamental problems of EC-coupling, for example, Ca^{2+} release termination, are discussed thoroughly in the recent review by Stern and Cheng (35).

4. The essence of the heartbeat is an SR generated Ca^{2+} oscillation

4.1. Entrained physiological Ca^{2+} cycling in ventricular myocytes by externally triggered rhythmic AP's

Although not usually articulated as such, the normal duty cycle of a cardiac ventricular myocyte is, in fact, a synchronized intracellular Ca^{2+} oscillation that is

initiated by an AP in response to excitation of the cell membrane from an external source (Fig. 2A and Fig. 3A, left). In normal myocardium, the initial source of the rhythmic excitations is the SAN, and in the experimental realm of isolated cardiac ventricular muscle or single cells, the external source is an electrical stimulator that produces inward current to trigger an AP. Thus, SR-originated Ca^{2+} oscillations in a normally functioning ventricular myocyte are driven or entrained by the external rhythmical stimulation. The amplitude of the triggered Ca^{2+} oscillation within a given beat is determined by 1) the amplitude of I_{CaL} that effects CICR, 2) the SR Ca^{2+} load, and 3) the extent of restitution of SR Ca^{2+} cycling mechanisms following the prior SR Ca^{2+} depletion and RyR inactivation by the prior AP (36–38). Neither the SR Ca^{2+} load, nor its Ca^{2+} cycling restitution kinetics is fixed, but each changes in response to changes in i) the rate or pattern of the external AP's, which determine net cell Ca^{2+} balance; ii) the velocity and extent of SR Ca^{2+} pumping into the SR; and iii) the recovery from inactivation status of SR Ca^{2+} release mechanisms. Stimulation of β -adrenergic receptors (β -AR's), via cAMP-mediated PKA-dependent phosphorylation of the L-type Ca^{2+} channel and of SR Ca^{2+} cycling proteins, enhances Ca^{2+} flux into the cell, SR Ca^{2+} loading, the restitution kinetics of SR Ca^{2+} cycling, the removal of RyR inactivation, and the amplitude of SR Ca^{2+} release (20).

4.2. When disconnected from its AP entrainment, cardiac SR becomes a "free running" Ca^{2+} clock

Since the cardiac SR is "wired" to physiologically oscillate Ca^{2+} , the potential for spontaneous Ca^{2+} release is inherent in the design of this organelle. When disconnected from the surface membrane, ventricular myocyte SR is no longer entrained by rhythmic AP's, but becomes a "free running" Ca^{2+} clock and generates spontaneous, roughly periodic Ca^{2+} releases even in the context of physiologic intracellular $[\text{Ca}^{2+}]$ (24, 39–41). Numerous experimental strategies have been employed to disconnect the SR from its entrainment by the surface membrane.

Physical SR disconnection: Isolated cardiac SR vesicles (42), electrochemically shunted cardiac myocytes (43), and cardiac cell fragments in which the sarcolemma has been mechanically removed (24, 40) all exhibit such spontaneous SR Ca^{2+} releases. When $[\text{Ca}^{2+}]$ is clamped in skinned cardiac cells, a physiological concentration of cAMP substantially increases the Ca^{2+} release frequency, and amplitude as well (due likely to recruitment of additional RyRs to participate in the Ca^{2+} release) (44).

Functional SR disconnection: In cardiac preparations

Fig. 2. Ca^{2+} control of the duty cycles of both ventricular myocyte and SANC. A: In ventricular myocyte, AP initiated global Ca^{2+} release (transient) is activated and synchronized by Ca^{2+} influx via $\text{I}_{\text{Ca,L}}$. The Ca^{2+} transient activates myofilament motion. B: In SANC, high basal cAMP mediated, PKA -dependent phosphorylation controls internal Ca^{2+} cycling by pumping Ca^{2+} into SR via SERCA2 and local Ca^{2+} release (LCR) via RyRs , as well as Ca^{2+} influx via $\text{I}_{\text{Ca,L}}$. The thick red line indicates spontaneous Ca^{2+} cycling. The spontaneous LCR from SR is linked to diastolic depolarization via Ca^{2+} activation of inward NCX current. cAMP or cAMP -mediated, PKA -dependent phosphorylation of ion channels (dashed lines) affects AP's characteristics to become commensurate with a given rhythmic ignition rate commanded by rhythmic SR Ca^{2+} cycling (Ca^{2+} clock). Cell images are shown together with the respective schematic cell type specific AP shapes.

Fig. 3. Properties of local Ca^{2+} releases (LCR) and LCR-induced membrane depolarizations in ventricular myocytes following cessation of repetitive stimulation. A: Simultaneous recording of Ca^{2+} signals (Indo-1), cell shortening, and membrane potential. B: Relationship between inter-stimulus interval and the time to the first LCR (spontaneous wave) after stimulation was stopped in control (blue circles) and after $1 \mu\text{M}$ isoproterenol (ISO, red circles). C: Confocal line scan image of Ca^{2+} signals (Fluo-4) measured in ISO ($1 \mu\text{M}$) stimulated rabbit ventricular myocyte. Red line is the time course of normalized Fluo-4 fluorescence averaged along the scanning line.

with intact surface membranes, spontaneous SR Ca^{2+} releases can be detected non-invasively in the absence of experimental Ca^{2+} loading by disconnecting the internal Ca^{2+} cycling apparatus from externally driven AP's, that is, by a voltage clamp (45, 46); simply by discontinuing electrical stimulation (Fig. 3); or acutely switching from a higher to a lower stimulation rate. Spontaneous SR Ca^{2+} releases were demonstrated in isolated cardiac ventricular muscle and also in a variety of other cardiac tissue types (47) by monitoring the scattering of laser light incident upon the tissue (48–52): microscopic motion within unstimulated isolated tissue or in the intact heart (52) caused by Ca^{2+} -myofilament interaction due to spontaneous SR Ca^{2+} release (38) produces intensity fluctuations in the scattered laser light (48, 49, 51). Successful isolation of Ca^{2+} -tolerant, single rat ventricular myocytes confirmed the tissue laser light scattering studies, indicating that spontaneous SR Ca^{2+} release-driven, microscopic myofilament motion occurs in the absence of Ca^{2+} overload (defined below), when cells are not electrically driven by AP's (53, 54).

4.3. Spontaneous local Ca^{2+} releases of the Ca^{2+} clock are localized and roughly periodic

The local nature of spontaneous Ca^{2+} release in cardiac cells: In contrast to the synchronized, or “systolic,” SR Ca^{2+} release and myofilament displacement triggered by an AP, spontaneous SR Ca^{2+} release, or myofilament motion driven by it, occurs locally within cells and asynchronously among various regions of a cell, and, within cardiac tissue, exhibits spatio-temporal heterogeneity among cells (38). When it became possible to directly measure intracellular Ca^{2+} in intact single cardiac cells, variable types of such spontaneous, local SR Ca^{2+} releases (LCR's) at rest, that is, in the absence of AP's, were observed having varying degrees of synchronization of RyR activation/recruitment (38). Thus, LCR's range from Ca^{2+} sparks, the elementary Ca^{2+} releases (55, 56), which are too small to generate measurable myofilament motion, to Ca^{2+} wavelets or waves (Fig. 3C), which are more synchronized, but still occur spontaneously and locally, giving rise to Ca^{2+} elevations as the wave traverses the cell. The local nature of spontaneous Ca^{2+} releases with various degree of RyR recruitment, distinguishes it from global, synchronized SR Ca^{2+} release triggered by an AP via CICR. LCR's have been observed in all cardiac cell types, including cardiac muscle (38), Purkinje cells (57, 58), and nodal cells (described below in great detail).

Factors that determine periodicity and extent of synchronization of spontaneous Ca^{2+} releases: LCR's

generated by “free running” SR are roughly periodic (49, 54), and since they are thought to occur when luminal SR Ca^{2+} reaches a threshold level (36, 59, 60), the period of time required for a subsequent spontaneous SR Ca^{2+} release to occur following a given release is determined, in part, by the speed at which the SR becomes filled with Ca^{2+} to reach the threshold level (36, 37). A key principle in this regard is that like the synchronized SR Ca^{2+} release triggered by an AP, the restitution time for successive spontaneous LCR's is not fixed; rather, it varies with the Ca^{2+} available for SR to pump, the speed at which it pumps, and the activation status of RyR's. These factors are regulated by cAMP-mediated, PKA-dependent phosphorylation of L-type Ca^{2+} channels, phospholamban, RyR's, and likely others (20, 44, 61, 62).

The SR Ca^{2+} clock, when disconnected from its normal physiological cycling (no excitatory AP's and I_{CaL}) cycles Ca^{2+} very slowly under resting/steady-state conditions, taking a long time to reach the threshold Ca^{2+} load required for spontaneous release. Thus, LCR's occur at a low frequency. In rat ventricle and atria, dog atria, and Purkinje fibers (47), spontaneous SR Ca^{2+} cycling persists in the absence of electrical stimulation in normal bathing $[\text{Ca}^{2+}]$ (Ca_0), but the frequency of such spontaneous Ca^{2+} releases is very low, for example, <0.1 Hz in rat ventricles (Fig. 3B, “REST”). In other tissue cells, for example, rabbit (Fig. 3C) or cat ventricles, in which the SR Ca^{2+} load becomes depleted in the absence of electrical stimulation, LCR's can occur transiently following cessation of stimulation, even in the absence of Ca^{2+} overload (defined below), become damped and cease (48, 53, 63–66).

The extent to which LCR's become synchronized is, in part, defined by the cell and SR Ca^{2+} loading, and the activation status of RyR's. At sufficiently large Ca^{2+} SR load, Ca^{2+} sparks can trigger propagating Ca^{2+} waves (55). Figure 3C demonstrates, in a rabbit ventricular myocyte, in the presence of β -AR stimulation, the entire spectrum of synchronization of SR Ca^{2+} release, that is, from the fully synchronized AP-mediated Ca^{2+} release, to spontaneous Ca^{2+} waves, to Ca^{2+} sparks. Following cessation of electrical stimulation, spontaneous Ca^{2+} waves transiently occur, followed by clusters of sparks, and then individual sparks followed by quiescence, when Ca^{2+} sparks are no longer detectable. The transient, spontaneous sparks following cessation of electrical stimulation are thought to provide a pathway for SR Ca^{2+} leak restricting SR Ca^{2+} content (67).

4.4. Free running SR status. Spontaneous (diastolic) SR Ca^{2+} release in ventricular tissue/cells reports the inotropic state

During steady rhythmic stimulation, cardiac cells tune their restitution mechanisms to match the demand of contractile performance. During normal heart beating, repeated SR Ca^{2+} depletion of ventricular cells by rhythmically occurring AP's emanating from the SAN resets the SR's rhythmic clock. Depleting the SR Ca^{2+} load at the onset of systole under physiological conditions thus prevents the occurrence of spontaneous SR Ca^{2+} release in diastole between AP's (Fig. 3). Experimentally, the rate of regular external stimulation required to suppress spontaneous SR Ca^{2+} release in cardiac ventricular preparations in a given scenario depends upon the speed at which the SR Ca^{2+} cycling clock restitutes, which in turn, depends upon the experimental conditions. If the subsequent AP is delivered prior to the completion of restitution required to reach threshold for spontaneous Ca^{2+} release, LCR's will not occur (Fig. 3). An abrupt discontinuation of external electrical stimulation allows complete restitution of spontaneous release mechanisms. The status of the entrained SR Clock can be interrogated by measuring the time from the last AP-induced, Ca^{2+} release to the first LCR (Fig. 3B). Acutely slowing the rate of electrical stimulation also permits the SR Clock to continue to run longer following the prior AP, and when its restitution time is less than the interval between successive AP's, spontaneous release occurs in the form of diastolic LCR's (63 – 65, 68).

Physiologic interventions that produce an increase in the contraction amplitude by effecting an increase in cell and SR Ca^{2+} loading, that is, β -AR stimulation (Fig. 3: B and C), impart a memory to the Ca^{2+} clock and reduce its restitution time. In other words, β -AR stimulation "speeds up" the SR Ca^{2+} clock. Of note, there is a striking positive correlation between the wide range of graded positive inotropy effected by gradations in perturbations that increase cell and SR Ca^{2+} , and there is a gradual reduction in time to the first LCR following a prior AP that these perturbations effect (38, 48, 63, 64, 68). However, when inotropic perturbations excessively increase the SR Ca^{2+} loading kinetics or Ca^{2+} load, the SR Ca^{2+} cycling clock runs faster than the surface membrane clock that produces regular AP's at a fired rate of electrical stimulation, LCR's begin to occur between AP's, and the inotropic state saturates. This is accompanied by subtle contractile amplitude "alternans" (69). Increasing the stimulation rate overdrives the free running SR Ca^{2+} clock, prevents diastolic LCR's (Fig. 3B), abolishes contractile alternans, and the average contractile state can further increase.

4.5. So! What is Ca^{2+} overload?

There is a general misconception within the cardiac research field that a free running SR is synonymous with " Ca^{2+} overload". The aforementioned considerations indicate that this is surely not the case. So! What is true " Ca^{2+} overload"? Ca^{2+} overload occurs during regular external stimulation when further experimentally-induced increases in cell and SR Ca^{2+} loading speed up the SR Ca^{2+} cycling clock period sufficiently to enable multiple LCR's during the inter-AP interval. A true state of " Ca^{2+} overload" is present when spontaneous SR Ca^{2+} releases (often multiple in number) that occur between AP's cannot be suppressed by a further increase in the external stimulation rate. In Ca^{2+} overload, increasing stimulation rate, by further increasing the SR Ca^{2+} load, further increases the number of LCR's between AP's, rather than suppressing them. Contractile alternans become marked, diastolic Ca^{2+} rises, and the inotropic state deteriorates. In the absence of external stimulation, Ca^{2+} overload is manifested by persistent LCR's in cardiocytes of species that do not exhibit LCR's at rest. For example, in rabbit or cat ventricular myocytes, the post-stimulation decay of LCR's (Fig. 3C) can be prevented by creating Ca^{2+} overload (70, 71), for example, by blocking NCX or by altering the ionic flux of NCX via increasing Ca_o or increasing intracellular Na^+ . In cardiocytes of species that do exhibit LCR's at rest in a normal bathing Ca^{2+} , for example, rat and dog atria, ventricle, and Purkinje fibers and guinea-pig atria, Ca^{2+} overload is manifested by an excessive increase in the frequency of resting LCR's (54). This perspective on the true nature of " Ca^{2+} overload" is most often not understood even by "experts" within the realm of Ca^{2+} dynamics, and the term " Ca^{2+} overload" is often misapplied to a physiologically fast ticking SR clock, mismatched to the rate at which external AP's are delivered by the experimenter.

4.6. Spontaneous- Ca^{2+} -release-ignited-excitations of the cell surface membrane

In ventricular cells with an intact sarcolemmal membrane, the occurrence of spontaneous Ca^{2+} release usually produces a small depolarization at rest or between successive AP's (Fig. 3A, bottom trace) of the cell membrane via activation of an inwardly directed I_{NCX} . This does not usually initiate a spontaneous AP in the cells, due, in part, to the presence of an outwardly directed current (I_{K1}), which stabilizes the diastolic membrane potential. When the SR clock ticks faster, LCR's occur more often during a given observation period, that is, the ensemble of LCR's within (Fig. 3C) and among cells becomes partially synchronized (multifocal spontaneous Ca^{2+} release), and this exposes

Fig. 5. Evolution of ideas of the cardiac pacemaker mechanism. Initial "polarized" concepts highlighting either strictly a subcellular or a membrane-delimited origin of cardiac pacemaker automaticity, for example, Bozler's 1943 "metabolism" oscillator and the 1962 Noble's model (Panels C and A, respectively). B: Schematic representation of a recent, membrane current-driven Kurata et al., 2002 model. D: A novel mechanism of normal pacemaker function: Interaction of "membrane clock" (external loop) and intracellular " Ca^{2+} clock" (internal loop) results in robust normal automaticity. I_{CaL} activation is common event for both loops.

a greater area of the sarcolemma to high Ca^{2+} . The NCX inward current that is ignited by multifocal spontaneous Ca^{2+} release is often sufficient to depolarize the surface membrane to the level required to activate Na^+ channels (41), and to generate a spontaneous AP (Fig. 4A). Thus, this phenomenon can be characterized as spontaneous- Ca^{2+} -release-ignited-excitations (SCaRIE). In the non- Ca^{2+} overload state, increasing the stimulation rate suppresses SCaRIE because it suppresses LCR's. In this case, SCaRIE is not "scary". However, in Ca^{2+} overloaded states, SCaRIE can initiate lethal ventricular arrhythmias. In this context SCaRIE is indeed "scary", and this scenario can be produced experimentally by intense β -AR stimulation when PKA-dependent effects on SR Ca^{2+} cycling in ventricular cells are not accompanied by an "adequate" increase in the external stimulation rate (that usually occurs in nature when cardiac ventricular β -AR are stimulated *in vivo*).

5. Early explorations of excitability of cardiac pacemaker cells

Cardiac muscle cells contracting in unison generate a mechanical force to pump blood. The next important question is what drives the rhythmic excitation of the cells within the heart? In other words, what is the mechanism of cardiac automaticity?

5.1. The myogenic origin of cardiac automaticity

The initial perspectives of the origin of cardiac automaticity focused on myogenic mechanisms. In the early 1880s, Gaskell, who conducted experiments applying heat to the tortoise heart, demonstrated that heart automaticity was myogenic in origin (rather than neurogenic). He noted that importantly, the automaticity could occur in all different regions of the heart including the ventricle (72), indicating that, in contrast to skeletal muscle, which strictly followed the nerve-mediated excitation $\rightarrow$ contraction paradigm, cardiac tissues possess their own internal excitatory oscillator. The area of the turtle heart with the greatest automaticity was the sinus venosus, which emanated impulses that entrained the entire heart.

5.2. Pharmacologic induction of spontaneous impulse initiation and self-sustained beating of isolated cardiac tissue

Evidence that an oscillatory intracellular process can initiate both normal and abnormal cardiac rhythms, in fact, stems from the turn of the last century (73, 74). The initial pharmacological experimental approaches towards understanding normal automaticity produced "brute force" Ca^{2+} overload, although this would not

be recognized until some 60–70 years later. Thus, abnormal rather than normal automaticity was being explored in early studies. Nonetheless, these studies set the stage for the later rejuvenation of hypothesis that intracellular Ca^{2+} is a partner with surface membrane channels in the process of normal automaticity.

Early electrographic and sphygmograph recordings indicated that abnormal excitation could be provoked pharmacologically and could come from basically any part of the heart. "Irregularity" of heart contractions induced experimentally by digitalis was reported in dogs as early as in 1897 by Cushny (75). Extrasystoles induced by digitalis, barium, or veratrine were then the focus of numerous studies in Germany and France in the first four decades of the twentieth century (73). Wenckebach coined the term "pararrhythmia" as a manifestation of activity of two independent foci. In 1912, Lewis (76) reported that premature beats, true paroxysmal tachycardia, and fibrillation have a common pathologic basis: "They are one and all the outcome of new impulse formation in the heart." His view was that, in paroxysmal tachycardia, a new "centre" of repetitive impulse formation initiates responses at a rate surpassing that of the normal rhythm and then "dominates the movements of the whole heart" (see ref. 10 for a review).

In 1929, Scherf showed that intravenous administration of aconitine produced accurately coupled atrial or ventricular extrasystoles of constant shape (77). Using a myographic recording, Segers studied contractions of turtle ventricle strips suspended in a divided chamber and identified the conditions that unleashed the internal oscillator of isolated cardiac muscle. If the end of the strip exposed to a normal perfusate were driven at a critical rate, the other end, if exposed to epinephrine, Ba^{2+} , or elevated Ca_o , suddenly became rhythmically active, driving the entire strip (78, 79). His recordings of monophasic potentials (i.e., between a normal area and an area in high KCl) demonstrated afterhyperpolarizations and early and delayed afterdepolarizations (80). Segers described this triggered muscle automaticity as "self-sustained beating" (81), and he pointed out that extrasystoles detected in electrocardiograms could be induced by the same agents that enhance afterdepolarizations, thus indicating that afterdepolarizations could be the cause for extrasystoles.

5.3. Concept of an intracellular chemical oscillator within cardiac cells

In 1943, working with rhythmically active strips of cardiac muscle (turtles, rabbits, cats, and dogs) and sinus venosus (turtle, high K^+) Bozler (82) noted oscillatory monophasic subthreshold potentials of relatively (compared to nerve) low frequency of approximately 1 Hz,

which were enhanced by increasing Ca_o or by adding adrenaline. Discharging impulses on the top of oscillatory potentials were accompanied by synchronized variations in isometric force (tonus) (83), indicating (to the present reader) that what he observed in 1943 was, in fact, SCA_{RIE}, and oscillations were, in fact, manifestation of “free running” SR Ca^{2+} clock under Ca^{2+} overload (Fig. 1R). Further, an important idea postulated at that time was that the observed oscillatory phenomena might be somehow linked to cardiac automaticity (83). Specifically it was postulated that “the tonus changes and the local potentials are probably manifestations of a more fundamental process, a fluctuation in resting metabolism... Their chief interest lies in their relation to the automaticity and rhythmicity of the muscle. It may be assumed that an increase in metabolism causes a rise in tonus and a decreased surface polarization. The decrease in polarization in turn may be considered as the last link in the chain of processes leading to the discharge of an impulse.” (83) (the concept is illustrated in Fig. 5C).

5.4. Concept of slow diastolic depolarization

A slow potential change preceding the discharge of cardiac impulses was first observed by Arvanitaki working on the snail heart in 1938. Later, in 1943, Bozler using monophasic potential recording found a similar phenomenon in sinus venosus of turtle (82). He called the slow depolarizing potential change a “pre-potential” and suggested that it was “the basis underlying automaticity” including “normal rhythmicity”.

The prepotential or slow spontaneous diastolic depolarization (DD), in current terminology, was later observed with microelectrode techniques by Brady and Hecht (84) in turtle hearts and by Coraboeuf and Weidmann (85) in spontaneously active Purkinje tissue. AP's from the SAN were first recorded by microelectrodes by West in 1955 (86). Microelectrode studies of SAN identified three important factors of pacemaker potential: the DD slope, the maximum diastolic potential, and the threshold for AP upstroke.

6. Evolution and dead end of dogma that pacemaker function is exclusively defined by a single specific ion channel current, “the pacemaker current”

Further successful development of quantitative membrane excitation theory by Hodgkin and Huxley in 1952 (The Nobel Prize in 1963) and its application to heart in 1960 (87), “stole the show” from the intracellular pacemaker mechanism as pacemaker field researchers focused nearly entirely on exploration of the membrane-delimited pacemaker mechanism, parti-

cularly on finding an ion current change underlying DD.

6.1. Application of the membrane current-driven voltage oscillator, derived from Hodgkin and Huxley theory, to cardiac pacemaker mechanism

Noble modified the Hodgkin-Huxley model to simulate both muscle (triggered) and pacemaker (spontaneous) cardiac AP's (87, 88). AP shapes, known from microelectrode studies, were reproduced in the model by an interplay of hypothesized voltage-gated and time-dependent Na^+ and K^+ currents. Although specific cardiac ion currents had not yet been measured at that time, there were some clues from the earlier microelectrode finding of a membrane conductance decrease during DD in pacing Purkinje fibers first reported by Weidmann (89) in 1951 and then, in beating sinus preparation of the rabbit's heart by Dudel and Trautwein (90) in 1958. The membrane response with various K_o and Na_o in the latter study suggested that the spontaneous depolarization was due to the decay of a specific K^+ conductance.

The Noble 1962 cardiac pacemaker model (88) had four current components, i_{Na} , i_{K} , i_{K1} , and $i_{\text{b,Na}}$, and the pacemaker mechanism was thus designed as the decline of g_{K} , which unmasked a background inward Na^+ current ($i_{\text{b,Na}}$), leading to membrane depolarization (termed “ g_{K} decay” mechanism, Fig. 5A). The 1962 cardiac modification of the Hodgkin-Huxley model was a mechanistic model based upon solid principles of thermodynamics of Bernstein's membrane theory and the Nernst's equation. The formulation of the 1962 model was a case of “theory leading experiment” (91): The membrane-delimited pacemaker mechanism, predicted by the theory, became the subject of extensive experimental search driven by successful application of the voltage clamp technique to various cardiac preparations (45, 46, 92, 93). Pacemaker specific ion currents were discovered and a variety of pacemaker mechanisms have been further suggested [see refs. 91, 94 – 97, for reviews].

6.2. Hard learned lessons of voltage clamp explorations of “the pacemaker current”, that is, the single most important current that governs pacemaker function

Purkinje cells: The search for the surface membrane cardiac pacemaker mechanism was initially and mainly studied, both experimentally and theoretically (from approx. 1950 to 1980), not in true pacemaker cells, that is, SANC, but rather in Purkinje fibers. A “pure K^+ selective” I_{K2} current redefined further as I_f , a “funny” non-selective monovalent cation current activated by membrane hyperpolarization (98), was acclaimed as the true “pacemaker current”. Retrospectively, there was a

price for the pacemaker research community to pay for the substantial investment made in this Purkinje fiber type. The “fundamental” pacemaker concept formulated in the 1962 model (88) and then revised in the McAllister-Noble-Tsien (MNT) model of 1975 (99), based upon Purkinje fiber voltage clamp studies, did not work for all pacemakers.

Sinoatrial nodal cells: Following the invention of the patch clamp method in 1976 by Neher and Sakmann (100) and isolation of single SANC by Taniguchi et al. in 1981 (93), further extensive voltage clamp studies have identified abundant ion current components in SANC, and new components are still being discovered. Many previously identified currents are split into sub-components; others are species dependent. For example, while SANC did exhibit the “funny” current, in most species, SANC had neither a fast Na^+ current nor an inward rectifying I_{K1} [see ref. 101, for review for species dependency]. The latter keeps resting potential low in Purkinje fibers, but not in SANC, so that I_{f} plays only a minor role in the pacemaker mechanism of SANC as its activation voltage is relatively low compared to maximum diastolic potential in the cells (91, 102). Furthermore, I_{f} has relatively slow (compared to DD duration in SANC) activation kinetics. It is not surprising that the block of I_{f} by Cs^+ had only minor effect on the pacemaker rhythm (103). It was also found that bullfrog sinus venosus cells lacked I_{f} (104), I_{Ks} is present

in porcine SANC, I_{Kr} is present in rat and rabbit (101), and I_{CaT} is almost absent in porcine SANC (105). Other currents were observed only in sub-populations of SANC (I_{Na} , I_{Cl}) (106) or had no obvious molecular identity (I_{st}) (107). As the result, net membrane current is currently viewed as being spread among numerous components and subcomponents (Table 1) and the fundamental (and thus rather simple) pacemaker mechanism claimed in early theoretical models has been lost. Several early (103, 108) and later studies (109), which specifically compared the contribution of different ion currents to the DD, concluded that there is no single pacemaker current in the SAN.

Currently there is no consensus on which of the SANC currents makes the major contribution to pacemaker activity, and the various view points include: 1) original “ g_{K} decay” mechanism of the 1962 model [I_{Kr} in rabbit (110)]; 2) I_{s} (I_{CaL} component) activation (111); 3) I_{f} activation (112, 113); 4) I_{CaT} activation (114); 5) I_{NCX} activation (115 – 117); and 6) I_{st} activation (107). Numerous numerical models, based upon gating schema of ensembles of SANC membrane ion currents, have been devised, and these models can indeed generate spontaneous AP’s (113, 118 – 123) (see example in Fig. 5B). Was there even a need to challenge the current membrane-delimited pacemaker dogma and to search for additional, intracellular mechanisms involved in pacemaker function?

Table 1. Ion currents reported in sinoatrial node cells of various species

Group of currents	Current	References
Voltage-gated Na^+ current	I_{Na} (absent in primary SANC)	211, 212
Voltage-gated Ca^{2+} currents ($\text{I}_{\text{Ca}} = \text{I}_{\text{CaL}} + \text{I}_{\text{CaT}}$)	I_{CaL} , High voltage-activated, L-type Ca current	213
	I_{CaT} , Low voltage-activated, T-type Ca current	114, 213
Voltage-gated K^+ currents ($\text{I}_{4\text{-AP}} = \text{I}_{\text{to}} + \text{I}_{\text{sus}}$ and $\text{I}_{\text{K}} = \text{I}_{\text{Kr}} + \text{I}_{\text{Ks}}$)	I_{to} , 4-AP sensitive transient K^+ current	214, 215
	I_{sus} , 4-AP sensitive sustained K^+ current (the sustained part of initially discovered I_{to} or $\text{I}_{4\text{-AP}}$)	215, 216
	I_{Kr} , the rapidly activating component of I_{K} exhibiting strong inward rectification (mouse, rat, guinea pig, rabbit)	217, 218
	I_{Ks} , the slowly activating component of I_{K} exhibiting only weak rectification (guinea pig, pig)	218
	I_{K1} , inwardly rectifying K^+ current (mouse, rat, and monkey)	101
Voltage-gated monovalent cation non-selective currents	Hyperpolarization-activated, “funny” current, I_{f} or I_{h}	96, 98, 112, 113, 171, 172
	Sustained non-selective current I_{st}	107
Background and ion transporter currents	Background Na^+ current, $\text{I}_{\text{b,Na}}$	219
	Background K^+ current, $\text{I}_{\text{K,Ach}}$	220
	Na^+/K^+ pump current, I_{NaK} or I_{p}	221
	$\text{Na}^+/\text{Ca}^{2+}$ exchanger current, I_{NaCa} or I_{NCX}	115, 116, 117, 153, 222
	Cl^- current, I_{Cl}	106
	Present in one third of SANC	

7. An alternative pacemaker mechanism initiated by an intracellular oscillator

But, could it be that not all of the crucial mechanisms that are implicated in spontaneous excitation of pacemaker cells are embodied in this ensemble of cell surface ion channels? Moreover and very importantly, could it be that the formal cause of spontaneous rhythmic AP's in SANC, that is, the initiating step of their normal automaticity, is an intracellular process?

7.1. The realization of the potential importance of Ca^{2+} in spontaneous AP's

The idea that an intracellular oscillator drives membrane excitations was temporarily revived as intracellular Ca^{2+} came into focus only in late 1970s – early 1980s. Unfortunately these studies continued to employ “brute force” Ca^{2+} overload in their approach to the problem. Pharmacological microelectrode studies of AP's in the early 1970s (73) rediscovered the “triggered activity” described approximately 30 years earlier in the monophasic recordings (80 – 83). It was shown that the AP's of Purkinje fibers exposed to ouabain or to acetylthiocholine develop “delayed afterdepolarizations” and aftercontractions; and the amplitude of these was enhanced by driving the fiber more rapidly (124 – 127), by increasing Ca_o , or by decreasing K_o and was depressed by Mn^{2+} (127). Intracellular Ca^{2+} had come into focus by this time, and based upon experimental observations that cardiac glycosides, a reduction in Na_o , or an elevation in Ca_o cause spontaneous contractions, it was suggested that an elevation of intracellular Ca^{2+} drives these spontaneous contractions. It thus became apparent that intracellular Ca^{2+} was involved in triggered activity or abnormal automaticity. The concept was further supported by the knowledge of oscillatory Ca^{2+} activity in a wide variety of cells (128). Data in cardiac cells, including Ca^{2+} dependent tension oscillations in skinned fibers by Fabiato and Fabiato (129) and voltage noise analysis by DeFelice and DeHaan (130, 131) in chick heart cell aggregates lead to the hypothesis that it is Ca^{2+} that provides the oscillatory substrate in the previously suggested intracellular “metabolic” oscillator (83).

7.2. Oscillatory Ca^{2+} -dependent (SR-driven) ion current under voltage clamp in cardiac cells

In contrast to the idea of an intracellular oscillator, interpretations from microelectrode studies suggested that spontaneous depolarization, that is, the membrane voltage change, *per se*, initiates internal Ca^{2+} release that drives spontaneous cardiac contraction (126, 127, 132, 133). However, microelectrode recordings of the

membrane potential do not allow assessment of the underlying ion currents, and this interpretation was disproven by voltage clamp studies that demonstrated a subcellular origin of the oscillations. Oscillatory current fluctuations under voltage clamp were observed in Purkinje fibers (134 – 138), as well as single Purkinje cells (139) and SAN (140). Both current and mechanical fluctuations are enhanced when intracellular free Ca^{2+} is elevated by bathing solutions containing high Ca^{2+} , low Na^+ , low K^+ , or toxic concentrations of digitalis. The power spectra of the currents showed peaks at oscillatory frequencies near 1 Hz at a room temperature (141), and the current oscillations were correlated with spontaneous contractile fluctuations. Finally, the oscillatory currents in Purkinje fibers disappeared with removing extracellular Ca^{2+} or chelating intracellular Ca^{2+} with injected EGTA (138).

Based on voltage clamp data, Kass and Tsien in 1982 (138) proposed that the intracellular oscillatory mechanism involves cycles of Ca^{2+} movement between SR and myoplasm, as previously suggested for skinned cardiac preparations by Fabiato and Fabiato (24). Since the oscillatory currents were inhibited by D600, a Ca^{2+} current blocker, a new and important idea was that Ca^{2+} oscillations represent the result of “a two-way interaction between surface membrane potential and intracellular Ca^{2+} stores” (138). The Ca^{2+} signals, including Ca^{2+} oscillations, were then indeed measured directly by aequorin in Purkinje fibers (30, 142).

An important issue that needed to be addressed was how Ca^{2+} oscillatory signals are transformed into the respective electrical signals of cell membrane? At that time it had been already discovered that ion pumps and exchangers can generate electric currents and that these, as well as ion channels, interact with intracellular processes. It turned out that major ion (K^+ and Ca^{2+}) currents in cardiac cells, including SANC, were modulated by Ca^{2+} and cyclic nucleotides. Based on this knowledge, it was speculated that intracellular Ca^{2+} oscillations could be linked to membrane potential via 1) a Ca^{2+} -dependent K^+ conductance (94); 2) a nonselective (Na^+ and K^+) cation channel (138, 143); or 3) I_{NCX} (91).

7.3. Realization of the “abnormal” flavor of the internal Ca^{2+} oscillator: the concept was not further pursued for normal pacemaker function

As noted, in order to demonstrate Ca^{2+} -driven oscillatory currents by techniques available during the late 1970s, Ca^{2+} -overload was produced in most experiments. Thus the prevailing assumption of the pacemaker field “Illuminati” at the time was that the oscillating current that became manifest to them under the Ca^{2+} overload state was a characteristic of “abnormal”

conditions/function, similar to that described by Lewis, Rothberger, Scherf, Segers, and Bozler in their studies (73). In these earlier studies, Ca^{2+} overload (probably not realized by these investigators) was employed to disengage the intracellular Ca^{2+} oscillations from their normal occurrence within the excitation-contraction paradigm in cardiac muscle. Despite the fact that it had already been demonstrated that similar fluctuations can also appear quite prominently in some heart cells in the absence of overt intervention (see above for detail), Kass and Tsien (138) discussed current fluctuations primarily as a disturbance of the normal cardiac function. In his 1984 review (91), Noble, upon testing an integration of a simple Ca^{2+} oscillator scheme into a pacemaker model, concluded that clarification of these abnormal rhythm mechanisms required additional experiments. Other pacemaker cognoscenti of the day, although basically in agreement with this view, were somewhat more liberal in their vision. Cranefield (73) reasoned that the true automaticity of the SAN or of Purkinje fibers might have a close connection with triggered activity. Irisawa speculated that subthreshold oscillations contribute to a “safety margin to prevent the SA node cell from becoming quiescent” (94). However since the spontaneous, localized, oscillatory Ca^{2+} signals in SANC could not be measured at the time, the concept that an internal Ca^{2+} oscillator provides the initiating signals for normal automaticity was again abandoned. But not for long! Because experimental procedures investigating the role of Ca^{2+} in pacemaker function shifted from the experimental Ca^{2+} overload paradigm to more physiologic conditions.

8. The physiologic intracellular Ca^{2+} clock within pacemaker cells is rejuvenated in late 1980s

8.1. Studies in Purkinje cells, atrial pacemakers, and in amphibian toad pacemaker cells

Afterdepolarizations and contractions were recorded in Purkinje fibers under normal Ca^{2+} loading conditions (144), and these fibers demonstrated localized spontaneous myofilament motion caused by LCR's (47). It was discovered that ryanodine (depleting SR content) has a profound negative chronotropic effect on automaticity of subsidiary atrial pacemakers (145). Analyses of the ryanodine effect on AP shape led to the suggestion that Ca^{2+} released from the SR contributes to DD, primarily during its later half, and plays a prominent role in bringing the late pacemaker potential to the AP activation threshold. Further studies of abnormal automaticity under conditions of Ca^{2+} overload indicated that Ca^{2+} release interacts with cell membrane by stimulating I_{NCX} (146). The importance of cellular Ca^{2+} and I_{NCX} for

normal spontaneous beating was also demonstrated in amphibian toad pacemaker cells (147).

8.2. Studies of intracellular Ca^{2+} involvement in SANC normal automaticity

Additional studies made a compelling argument that Ca^{2+} cycling within SANC, the cells of primary pacemaker tissue, is crucial for their normal automaticity. Ca^{2+} cycling proteins, SERCA, RyR, NCX, previously identified in atrial or ventricular cells, were also identified in SANC (148). A possible importance of SR Ca^{2+} release and related I_{NCX} for SANC pacemaker function was demonstrated by the ryanodine effect in these cells (115–117). When it became possible to routinely measure intracellular Ca^{2+} in SANC, it was observed that a cytosolic Ca^{2+} transient is evoked by the AP and that Ca^{2+} influx via L-type Ca^{2+} channels affects Ca^{2+} loading of the SR; chelation of intracellular Ca^{2+} in rabbit SANC by BAPTA (116, 149) [but not EGTA! (149, 150)] applied intracellularly, markedly slowed or abolished spontaneous SANC beating (Fig. 6A). A strong negative chronotropic effect of EGTA, however, had been reported in AV node cells of guinea pig (151) and in guinea pig SANC (152). Changes in the cytosolic Ca^{2+} transient in response to β -AR stimulation were correlated with changes in the beating rate (147, 148). Thus, it was becoming recognized that intracellular Ca^{2+} cycling within pacemaker cells is somehow involved in normal SANC pacemaker function.

8.3. Local subsarcolemmal Ca^{2+} releases in pacemaker cells during diastolic depolarization

Confocal Ca^{2+} imaging permitted detection of a more subtle form of spontaneous Ca^{2+} releases than those caused by experimental Ca^{2+} overload. Recent studies in rabbit SANC employing confocal imaging show that in contrast to ventricular myocytes, the SR Ca^{2+} clock is in part entrained by an AP but later in the cycle becomes “free running”. The complete cycle of “free running” Ca^{2+} clock is illustrated in Fig. 5D by an internal loop. During spontaneous beating, LCR's occur beneath the cell membrane during late DD in the form of multiple sub-membrane Ca^{2+} sparks and/or small Ca^{2+} wavelets (153) (Fig. 4B). Additional robust experimental evidence indicates that in the basal state, such LCR's are frequent and powerful enough to initiate the SANC duty cycle and thereby sustain robust normal automaticity (153–157). Ca^{2+} release-excitation coupling (Fig. 1E) and S Ca RIE (Fig. 4B) are initiated by LCR's that ignite significant NCX inward current (Fig. 6: C–H), which imparts an exponential increase to the later part of DD, prompting activation of surface membrane L-type Ca^{2+} channels to initiate an AP (153,

Fig. 6. Ca^{2+} controls the pacemaker rate of rabbit SANC by activation of inwardly directed NCX current. A, Effects of BAPTA-AM and EGTA-AM on frequency of spontaneous AP's in SANC. A: AP's were recorded before (Control) and after superfusion with $5 \mu\text{M}$ BAPTA or $30 \mu\text{M}$ EGTA for 5 min. B: Average response of AP frequency in cells treated with EGTA-AM ($n=7$) and with BAPTA-AM ($n=8$). $*P<0.05$ vs control and EGTA groups. C: Dose dependence of the ryanodine inhibitory effect on the spontaneous AP firing rate. D: Linescan image of Ca^{2+} release with superimposed AP records in control (Ctrl) and 1 min after addition of $3 \mu\text{M}$ ryanodine (ryan). E: Ryanodine inhibits $[\text{Ca}^{2+}]_i$ release and inward current during DD without affecting peak I_{CaL} . Normalized fluorescence (top) and membrane current (left middle) under voltage clamp before and 4 min after addition of $3 \mu\text{M}$ ryanodine; voltage-clamp protocol is shown in bottom panel. Middle right panel displays indicated part of current record at greater magnification. *: Local $[\text{Ca}^{2+}]_i$ increase inhibited in the presence of ryanodine; #: corresponding inward current inhibited in the presence of ryanodine. F–H: Representative examples of inhibitory effect of Na^+ replacement by Li^+ on spontaneous beating in SANC. F: AP recordings during onset and after washout of Na^+ -free, Li^+ -containing solution. G: 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 curve 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. H: Normalized fluorescence (top), and membrane current (middle) during voltage clamp before (black) and 10 seconds after (" Li^+ ") superfusion with Na^+ -free, Li^+ -containing solution during voltage protocol (bottom). Highlighted area shows an inhibitory effect of Na^+ -free Li^+ spritz on inward current during the voltage ramp. Panels A and B from ref. 149. Panels C–H from ref. 153.

indicative of a physiologic "free running" SR (see above) and is typical of rabbit and cat, and some other species, as well. In SANC voltage clamped at near the NCX reversal potential, the rhythmicity of LCR's and the current fluctuations that these generate can be demonstrated (155, 156).

9. Interactive membrane and submembrane Ca^{2+} cycling in SANC is tightly controlled by basal phosphorylation: a novel mechanism of normal pacemaker function

The critical question is then why SANC generate LCR's in the basal state? A simple explanation might be that Ca^{2+} levels are higher in SANC than those in ventricular myocytes. However this idea was disproven by direct measurements of $[\text{Ca}^{2+}]$ levels in spontaneously beating rabbit SANC (155, 157), which indicated that Ca^{2+} levels in SANC are similar to those in rabbit ventricular cells (159).

A recent discovery provides a clue for why the basal Ca^{2+} clock operation is greatly enhanced in SANC: Basal levels of cAMP and cAMP-dependent phosphorylation within SANC are high, that is, even in the

157). Thus SCAIE initiates the SANC duty cycle.

While LCR's occurring in cat latent atrial pacemaker cells are thought to be voltage-dependent, as these appear to be triggered by Ca^{2+} (by CICR mechanism) entering the cell via T-type Ca^{2+} channels activated by the DD (158), in spontaneously firing rabbit SANC, LCR's are truly spontaneous and are not triggered by the depolarization. Rhythmic LCR's occur in SANC in the absence of membrane potential changes, that is, during voltage clamp (at -10 mV to prevent SR Ca^{2+} from depletion) or following "chemical skinning" of the surface membrane with the detergent saponin (155, 156) (Figs. 7 and 8, panels a and c, Control). When SANC are voltage clamped at the maximum diastolic potential, the SR Ca^{2+} clock runs for a few cycles and then stops due to SR Ca^{2+} depletion (Fig. 9A, i) (155). This decay and cessation of LCR's when AP's cease is

absence of β -AR stimulation (Fig. 10). The high levels of basal cAMP are due to high constitutive adenylyl cyclase activity that is not driven by constitutive β -AR activation (156). Preliminary evidence indicates that two adenylyl cyclase isoforms: type 2, a Ca^{2+} insensitive isoform, and type 8, a Ca^{2+} -activated isoform, highly expressed in the brain, are expressed in SANC, but not in other cardiomyocytes (160). Interestingly, basal phosphodiesterase activity also appears to be elevated in

SANC (161), likely as a mechanism to keep basal cAMP levels in check.

The rate at which the Ca^{2+} clock runs is thus under tight physiologic regulation by the degree of PKA-dependent phosphorylation. This determines the Ca^{2+} available for SR pumping and modulates functions that govern the velocity of Ca^{2+} pumping and the activation of the RyR's by internal SR Ca^{2+} . Among SANC proteins highly phosphorylated in the basal state and implicated in enhanced SR Ca^{2+} cycling are phospholamban (Fig. 10) and RyR's (156). In skinned or voltage clamped SANC, inhibition of PKA-dependent phosphorylation by PKI reduces and abolishes LCR's, indicating a crucial role of PKA-dependent phosphorylation in the regulation of the Ca^{2+} clock.

Fig. 9. Local Ca^{2+} releases in voltage clamped rabbit SANC. A: i: confocal linescan image and normalized sub-sarcolemmal fluorescence averaged over the image width in a representative SANC. Double-headed arrows delineate cycle length ("Cycle") and the LCR period ("LCR") during spontaneous beating and during the first would-be cycle during voltage clamp. ii: Closed squares, relationship between cycle length and the LCR period during spontaneous beating (10 cells, 86 LCR's); open squares, relationship between LCR period and the first would-be cycle during voltage clamp (13 cells, 17 LCR). B: Simultaneous recovery of LCR period and cycle length after removal of voltage clamp. i: Simultaneous recordings of AP's (top) and linescan image in a representative SANC during recovery from voltage clamp. Arrow indicates the moment of voltage-clamp removal. ii: Average relative recovery of total signal mass of LCR's, cycle length, and DD rate after removal of voltage clamp ($n = 5$). Recovery of total signal mass of LCR's and DD rate were highly correlated ($R^2 = 0.78$), as were the recovery of total signal mass of LCR's and spontaneous cycle length ($R^2 = 0.92$). iii: Triangles, the recovery of the LCR

period and that of the cycle length after removal of voltage clamp (triangles) were highly correlated ($R^2 = 0.86$); closed squares, relationship between cycle length and the LCR period during spontaneous beating (same as in panel A, ii). Dashed line is the line of identity. From ref. 155.

Fig. 10. High basal level of cAMP and PKA-dependent phospholamban (PLB) phosphorylation in SANC. a: Average content of cAMP in suspensions ($n = 4$, each) of SANC, atrial or ventricular myocytes, and changes in cAMP level in SANC following suppression of AC activity with $400 \mu\text{M}$ MDL ($n = 3$) or β -AR stimulation ($1 \mu\text{M}$ ISO, $n = 3$). b: Left, Western blots of the basal level of PLB phosphorylated at serine¹⁶ and total PLB in SANC and ventricular myocytes; right, average values of phosphorylated PLB normalized to total PLB ($n = 4$). c: Phosphorylated PLB and total PLB at baseline 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 ($15 \mu\text{M}$ PKI), β -AR stimulation ($1 \mu\text{mol/L}$ ISO). e: Average changes in phosphorylated PLB normalized to total PLB induced by maneuvers in c and d ($n = 3$ in each group, MDL concentration was $400 \mu\text{M}$). * $P < 0.05$. From ref. 156.

lation of SR Ca^{2+} cycling proteins in the generation of basal spontaneous LCR's in SANC (156) (Fig. 7). In addition to phospholamban and RyR's, basal PKA-dependent phosphorylation of numerous other proteins, yet to be identified, likely participate in functions to insure that the Ca^{2+} clock runs at a sufficient rate to produce basal LCR's. The frequency and signal mass of LCR's increase when cAMP is added directly to skinned cells or when β -AR's are stimulated during voltage clamping (156) (Fig. 8). This observation explains the major positive chronotropic effect of β -AR stimulation in SANC in new terms: the stimulation extends the range of PKA-dependent control of the Ca^{2+} clock speed that ultimately determines the enhanced rate at which the surface membrane clock becomes ignited by LCR's (154, 156).

9.1. Mutual entrainment of Ca^{2+} and membrane clocks in SANC optimizes pacemaker operation at a given rate: SCARIE is not "scary"

The Ca^{2+} cycling proteins of the SR, the NCX, L-type Ca^{2+} channels, and other sarcolemmal ion channels, mutually entrain and thus tightly control each other during spontaneous basal and β -AR-stimulated SANC firing. This entrainment results in stable/robust pacemaker operation at a given pacemaker rate. Multiple control points/feedback loops are mediated by the functional dependencies of the involved proteins on Ca^{2+} , cAMP, phosphorylation, and membrane potential (Fig. 2B and Fig. 5D).

The key role of I_{CaL} and NCX: In contrast to ventricular myocytes, the electrical excitation in primary SANC is mainly mediated via activation of I_{CaL} rather than I_{Na} . In addition to being a component of the membrane clock, I_{CaL} is also a crucial factor in the SR Ca^{2+} clock (it couples internal and external oscillatory loops in Fig. 5D). The relatively synchronous SR Ca^{2+} release triggered by I_{CaL} causes a global SR Ca^{2+} depletion that quenches the spontaneous LCR's and resets the SR Ca^{2+} clock, as in paced ventricular cells. Moreover, Ca^{2+} influx via L-type Ca^{2+} channels also "rewinds" the Ca^{2+} clock by supplying Ca^{2+} to be pumped into the SR. Thus, LCR's, by entrainment of rhythmic NCX and L-type Ca^{2+} channel activation, entrains the membrane clock to produce rhythmic AP's. The magnitude of L-type channel Ca^{2+} influx is finely tuned by the channel inactivation by the cytosolic Ca^{2+} transient that it triggers. During the AP, the electrogenic NCX ion flux is finely tuned by the membrane depolarization induced by L-type Ca^{2+} channels and by membrane repolarization due to K^+ channels. Thus, in the process of entrainment of L-type channels and its Ca^{2+} influx, the LCR clock entrains itself.

In this regard, it is important to note that the basal SANC Ca^{2+} clock function is supported by an enhanced L-type Ca^{2+} channel activity, as these channels seem to be highly phosphorylated at the basal state by PKA, since PKA inhibition suppresses more than 50% of I_{CaL} (162). I_{CaL} in SANC is also regulated by CaMKII-mediated phosphorylation: it regulates I_{CaL} inactivation and reactivation, and local Ca^{2+} is critically involved in these processes (149).

Other ion channels indirectly support Ca^{2+} clock operation: Four other ion currents in SANC, I_f , I_{CaT} , I_{Cl} , I_{Kr} , $I_{\text{Na,bkg}}$ (c.f. Table 1), were found to be, to a varying degree, Ca^{2+} dependent, and therefore, intracellular Ca^{2+} signals have these additional control points within the membrane clock. Although data on Ca^{2+} dependence of I_f is mainly controversial (163 – 165), the most recent study (166) proposed a role for Ca^{2+} and calmodulin in the regulation of I_f in SANC that is independent of CaMKII. An increase in $[\text{Ca}^{2+}]_i$ is known to increase I_{K} in ventricular myocytes (167, 168). β -AR stimulation increases I_{K} by causing a negative shift of the current activation curve and also increases the rate of deactivation of this current (169). A critical role of I_{K} modulation by PKA is to maintain an AP duration commensurate with a given firing rate (170). Regarding I_f channels, cAMP, a second messenger of β -AR stimulation, directly facilitates the opening of these channels, shifting the I_f activation curve to more positive voltages (171, 172). Another, non-selective "sustained" current, I_{st} , is also enhanced by β -adrenergic stimulation (107, 173), but the exact signaling pathway remains unknown.

Although modulatory interactions of Ca^{2+} and membrane clocks are rather complex and remain under-explored, modulatory changes of ion channel characteristics produced by Ca^{2+} , cAMP, and cAMP/PKA-dependent phosphorylation described above can be viewed as supporting the enhanced Ca^{2+} clock operation (dotted lines in Fig. 2B), as they tune and optimize SCARIE: 1) as already noted, a larger Ca^{2+} influx via I_{CaL} enhances the SERCA Ca^{2+} pumping rate (in addition to the phospholamban modulatory effect); 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 and increase in I_{st} limit the extent of hyperpolarization by the increased I_{Kr} , ensuring that LCR-activated I_{NCX} operates within the voltage range close to the I_{CaL} activation threshold. It is important to note that while ion channels tune the Ca^{2+} clock, changes in ion channels alone in response to PKA dependent mechanisms have a very limited chronotropic effect, as tested experimentally with ryanodine inhibiting LCR (154) (Fig. 11).

Fig. 11. Effects of RyR inhibition on the ISO-induced potentiation of DD current, I_{CaL} , and spontaneous SANC firing rate. A and C: Voltage protocols and original current recordings for the simulated DD current and I_{CaL} , respectively. B: Relative increase in mean DD current amplitude by 1 μ M of ISO before ($n = 5$) and after block of RyR with 3 μ M of ryanodine ($n = 4$). D: Relative increase in mean value of the I_{CaL} by 1 μ M of ISO before ($n = 6$) and after 10 μ M of ryanodine pretreatment ($n = 8$). * $P < 0.05$. E: Effects of RyR inhibition on the concentration response of SANC firing rate to β -AR stimulation. The relative increase in SANC firing rate in response to ISO is reduced by 3 μ M of ryanodine. ISO caused a dose-dependent increase in the firing rate with an apparent threshold concentration of 0.1 μ M. This effect was markedly decreased in the presence of ryanodine. The maximal effect of ISO occurred at 1 and 3 μ M in the absence and presence of ryanodine, respectively. Inset illustrates the shift of the relative ISO dose-response curve by ryanodine; the curves in the presence or absence of ryanodine are normalized to their own maxima. From ref. 154.

9.2. Testing control points of Ca^{2+} clock in SANC: experimental evidence for its ultimate importance for pacemaker function

Studies that have focused on surface membrane delimited pacemaker mechanism utilize voltage clamp techniques to measure ion channel currents and then attempt to establish a role for these channels in normal automaticity via extrapolation of voltage clamp data to numerical model simulations. In contrast, the approach to establish a role for LCR's in normal automaticity utilizes both spontaneously beating and voltage clamped SANC. Robust experimental evidence demonstrates a tight coupling among the speed at which the Ca^{2+} clock runs and the rate at which spontaneous AP's are generated.

1. Cycle length variations among individual SANC: The cycle lengths in individual SANC tightly correlate with their respective LCR periods, defined as the time from AP-triggered Ca^{2+} release to LCR emergence during DD (Fig. 9A) (155, 156).

2. Recovery from Ca^{2+} depletion: Voltage clamp at the maximum diastolic potential results in cessation of spontaneous beating because of SR Ca^{2+} depletion and LCR abolition. Returning to spontaneous beating from the voltage clamp is followed by the gradual recovery of the normal spontaneous beating rate, and this recovery is highly correlated with the return of LCR's and the

recovery of the control LCR period (Fig. 9B) (155).

3. Pharmacologic interventions: Graded, submaximal concentrations of nifedipine, ryanodine, or BAPTA produce gradual prolongation of the LCR period, that is, slowing of the Ca^{2+} clock and slowing of the spontaneous AP rate of single rabbit SANC. The prolongation of the LCR period and slowing of the spontaneous AP rate affected by these maneuvers are highly correlated (174). Higher concentrations of BAPTA or ryanodine block beating, as does substitution of Li^+ for Na^+ in the bathing fluid (153) (Fig. 6).

A novel aspect of pharmacologic intervention involves basal PKA modulation (Fig. 12). Submaximal PKA inhibition slows the Ca^{2+} clock and the spontaneous AP firing rate. The time-dependent effects on the spontaneous AP firing rate, of basal PKA inhibition and its reversal in individual, isolated SANC, are highly correlated with the concomitant time-dependent changes effected in LCR period (Fig. 12A). (156). After prolonged time, or following higher levels of these inhibitors, LCR's are no longer detectable, and the rate of spontaneous AP's markedly slows, becomes highly irregular and often ceases. 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. 12B) (156). Alternatively,

Fig. 12. Spontaneous cycle length in rabbit SANC is tightly linked to protein kinase A signaling. 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 LCR's is shorter than the spontaneous cycle length. B: (top), Typical example of AP's recorded in a single, isolated SANC before (Control), during superfusion with 15 μM PKI, and during washout of the drug. (bottom), The relationship of PKI-induced suppression of single nodal cell 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; triangle symbols depict the data simulated by numerical model using average experimentally measured changes in LCR characteristics and phase (see text for details). Modified from ref. 156.

increasing PKA-dependent phosphorylation by stimulating β -AR's or by adding cell permeable cAMP analogs to the bathing fluid reduces the LCR period (i.e., speeds up the Ca^{2+} clock) and increase the spontaneous AP firing rate and the two are highly correlated (Fig. 12C). Although augmentation of I_{CaL} is necessary for the cAMP-dependent effect to increase beating rate, it is not sufficient, because blocking RyR function, whilst leaving cAMP-dependent I_{CaL} augmentation intact, markedly reduces the cAMP-dependent increase of the SANC AP firing rate (Fig. 11). 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 forms a line of identity, Fig. 12D).

10. Ca^{2+} dynamics in numerical pacemaker models: past, present, and future

10.1. The fundamental failure of membrane-delimited pacemaker design

The 1962 model (88) and MNT models (99) assumed fixed ion concentrations and could not explain a variety of experimental observations, including Ca^{2+} dynamics. In the early 1980s, the essential problems of membrane-delimited approach became evident for the pacemaker field, and this prompted investigators to make a revolutionary move by including formulations for ion concentrations in their pacemaker models (91), following basically an approach undertaken earlier by Beeler and Reuter in 1977 (175) to introduce $[\text{Ca}^{2+}]$ into a ventricular myocyte AP model. While the upgraded Purkinje cell model [DiFrancesco-Noble model of

1985 (176)] and its SANC modification [Noble-Noble model of 1984 (118)] embraced ion balance [see refs. 177 and 178, for reviews], precise studies on Ca^{2+} dynamics in pacemaker cells have not been available until very recently, so that the model formulations have been utilizing experimental parameters determined in the “wrong” cells: the Ca^{2+} release equations were set to muscle cell type release (118) (Noble-Noble model 1984) that was initially thought to be both voltage- and Ca^{2+} -dependent.

Subsequent SANC models basically followed the development of Ca^{2+} release formulations in the models of cardiac muscle cells. The most complex SR kinetics formulations were developed in the model by Demir et al. 1994 (119) based on the modeling study of an atrial myocyte by Hilgemann and Noble in 1987 (179), including the internal SR Ca^{2+} -buffering by calsequestrin. As a result, in the SANC models, all Ca^{2+} release occurs during the onset of systole in the form of AP-induced global Ca^{2+} transient serving to EC-coupling, rather than to a pacemaker function. In other words, the upgraded SANC models reproduced neither spontaneous diastolic Ca^{2+} release nor intrinsic Ca^{2+} oscillations, so it is not surprising that their ion dynamics were more or less “decorative” and had negligible effects on the cell membrane (especially during the DD, see “ Ca^{2+} transient” in Fig. 5B). And *de facto*, spontaneous AP’s generated by these models are still gripped within the same original 1962 model paradigm, that is, the interplay of membrane-delimited ion currents (compare Fig. 5A and Fig. 5B). Realizing the ineffectiveness of ion dynamics for pacemaker function portrayed in this way, some models (120) greatly simplified the Ca^{2+} release formulations, and a more recent model (121) has assumed all ion concentrations fixed. This “back to the future journey” completes a 40 years pacemaker exploration cycle: it has finally landed at the very starting point, that is, back to 1962!

It is our opinion that there is a fundamental problem of membrane-delimited pacemaker designs of SANC, despite the inclusion of ion concentration terms in the majority of the models since more than two decades ago. The membrane-delimited (reductionist) approach assumes that following backwards integration, isolated current components (Table 1) will reproduce the final pacemaker function in almost its entire physiological integrity. However, the ion current characteristics are discovered under specific experimental conditions (e.g., voltage clamp, dialyzed whole cell, cocktails of blockers, ion concentrations, artificial ion buffers, cAMP, protein phosphorylation), each of which might differ from that in intact functioning pacemaker cells. When such data, obtained in the context of disrupted physio-

logic links, are incorporated into a variety of pacemaker model designs, the models simulate a satisfactory AP shape, but the nature of the net current change in the spontaneously beating cell *in situ* may be still different from that in the models.

Furthermore, and very importantly, a crucial modulatory effect of an intracellular event, that is, a “formal cause” of the membrane potential change that initiates an AP can be easily overlooked in the membrane-delimited modeling approach. Specifically, since the membrane electrical resistance during the DD is very high: a tiny current change of approximately 3 pA is enough to drive the critical DD change in rabbit SANC (113). A current change of this magnitude could be easily generated by ion current variance within experimental voltage clamp data. Thus, it could be misinterpreted as a current that is initiated by channels within the membrane, rather than one being generated by a modulatory effect on the membrane current, actualized by an intracellular event, such as spontaneous/oscillatory Ca^{2+} release during DD.

An argument that is often referred to favor a membrane-delimited pacemaker mechanism, and to discount a role for intracellular Ca^{2+} , is that the membrane current does not show cyclic changes under the voltage clamp in SANC (95, 97). However, this is only true with reference to a comparison with oscillatory current scaling of more than 100 pA, as observed in digitalis-treated ventricular myocytes, and interpreted as the “gold standard” of the “oscillatory current” (Fig. 1 of ref. 97). Further, accurate perforated patch clamp studies, which preserve crucial intracellular interactions within rabbit SANC, clearly demonstrate an oscillatory nature of the net membrane current. The oscillatory current traces measured under these new experimental settings show distinct peaks in their Fourier spectra with the dominating frequencies being slightly higher than the frequency of spontaneous AP’s in the cells (in Figs. 7 and 8, panels c–f “Control”) (155, 156). However, since the scale of the oscillatory current in SANC is only 10 pA, it could be easily distorted and missed in early patch clamp whole-cell experiments (95, 97).

Another argument often raised in favor of membrane-delimited ion channel-driven pacemaking is that pacemaker cells continue to generate spontaneous AP’s in the whole cell patch clamp configuration with EGTA inside the cells. More recent experimental data, in rabbit SANC, however, show that while EGTA is indeed ineffective in suppressing spontaneous AP’s, the fast Ca^{2+} -chelating buffer, BAPTA slows and stops SANC normal automaticity (149) (Fig. 6). This is consistent with theory predicting that EGTA is insufficient to

buffering local Ca^{2+} in confined spaces (180), such as the submembrane space where LCR's from SR "talk" to NCX.

Although knowledge of modulation of ion currents by intracellular messengers (such as Ca^{2+} , cAMP, phosphorylation), as discussed above, is crucial, it cannot substitute for knowledge about intracellular modulatory events *per se*, which could "live their own life" being relatively independent of the cell membrane (internal Ca^{2+} loop in Fig. 5D). The price paid for missing a fundamental role of critically timed, local submembrane Ca^{2+} release and its modulation by PKA-dependent phosphorylation is that SANC models (in Fig. 5A and 5B and alike) could not reproduce many pharmacological effects or β -adrenergic modulation. Thus, in the midst of evolving evidence about the influential interactions of the intracellular signaling with cell membrane in SANC, it becomes clear that the assumption of membrane-delimited pacemaker function may be fundamentally incorrect and that theoretical models of SANC operation with an integrated SANC-specific spontaneous diastolic Ca^{2+} release tightly controlled by basal phosphorylation need to be developed (153 – 157, 181).

10.2. Ca^{2+} clock-driven pacemaker mechanism is tested by new numerical modeling

Our analysis of the existing cardiac cell AP models revealed that the concept of spontaneous oscillatory Ca^{2+} releases driving roughly periodic AP's had indeed been previously considered. However, experimental paradigms utilized to generate data for these models were implemented in the Ca^{2+} overload state. Many cardiac numerical models including those for Purkinje cells (91, 178, 182, 183) and ventricular cells (184) reproduce spontaneous Ca^{2+} release ignited excitations, in the context of Ca^{2+} overload conditions. For example, in the 1985 DiFrancesco-Noble model of Purkinje fiber, for Ca^{2+} release to occur spontaneously and become repetitive, high intracellular $[\text{Na}^+]$ and related Ca^{2+} accumulation are required (183). Each Ca^{2+} release activates I_{NCX} which, if large enough, can trigger additional (ectopic) AP's, as shown in Fig. 13 (183).

It is our opinion that the Ca^{2+} cycling-triggered AP's, considered as abnormal for other cell types due to the Ca^{2+} overload conditions employed, are normal for SANC operation, as SR Ca^{2+} cycling is indeed enhanced at the basal state in this cell type (155, 156) when intracellular Ca^{2+} is within the normal range (157).

The most simple and direct test of whether submembrane LCR's occurring during the later part of the DD may indeed drive SANC pacemaker function was to introduce the Ca^{2+} waveform measured by confocal

Fig. 13. The Di-Francesco-Noble 1985 Purkinje fiber model manifests AP evoked by spontaneous Ca^{2+} release via activation of NCX current under sodium/calcium overload, $[\text{Na}]_i$ was increased from 8 to 12 mM. Reprinted with permission from ref. 183, ©2002 AAAS.

microscopy into existing numerical SANC models to override the model prediction for Ca^{2+} . Since Ca^{2+} was set constant in the Zhang et al. model (121), introduction of the real Ca^{2+} changes into that particular model was rather revealing. It turned out that after a short transitional process, the model accelerated its beating rate, adopting the rate of experimentally measured Ca^{2+} transients (181). This simple, initial test indicated that LCR's and related I_{NCX} that normally occur during the DD, indeed cannot be ignored, and under normal conditions, could be powerful enough to command the final pacemaker AP rate.

A recent SANC numerical model has included formulations of Ca^{2+} dynamics in the submembrane space (122) and a new model adopting these formulations has been developed to explore the function of spontaneous LCR's in rabbit primary SANC (157) (Fig. 14). This model, with novel diastolic Ca^{2+} release formulations, in contrast to previous models, predicts a variety of experimental findings, including the marked negative chronotropic effect of ryanodine (157). Another new important finding of the model was that both the spontaneous Ca^{2+} release rate from the submembrane SR to produce an LCR and phase of the Ca^{2+} release control the beating rate of the SANC, that is, the larger release allowed the wider range of rate regulation by varying the period (i.e., phase within SANC cycle) of the oscillatory release (Fig. 14: A – C). The range of rate regulation in the model covered the entire physiological range under normal conditions and under hormonal stimulation (Fig. 14C). Model simulations demonstrated that the LCR-activated I_{NCX} imparts an exponentially rising component to the late DD that culminates in an AP upstroke (185) as previous

Fig. 14. Novel numerical models of SANC spontaneous firing incorporate characteristics of the intracellular Ca^{2+} clock. **A:** a SANC model with introduced diastolic Ca^{2+} release. **B and C:** both the spontaneous Ca^{2+} release rate ($P_{rel,spont}$) from the junctional SR to produce an LCR and phase (t_{phase}) of the release control the beating rate of the SANC, that is, the larger release allowed the wider range of rate regulation by varying release phase within the duty cycle. **D:** Model simulations of chronotropic interventions with ISO or PKI. Membrane potential, NCX current (I_{NCX}) overlapped arbitrary eight LCR's, and average submembrane $[Ca^{2+}]$ are depicted. The respective model parameters are given in Table 2 of Online supplement in (156). **E:** The effect of enhancing or decreasing PKA-signaling on spontaneous cycle length. "PKI data" and "ISO data" bars show mean values of experimentally measured effects of PKI and ISO $\pm$ S.E.M., respectively; other bars show numerical model predictions with selected PKA targets (see labels at the bars) being activated or inactivated. Panel A from ref. 155, C modified from ref. 157, D and E from ref. 156.

experiments have shown (153, 174). The model also reproduced the scale of ion current oscillations observed under voltage clamp in SANC (155). Simulated sponta-

neous Ca^{2+} releases in SANC not only regulate the rate of spontaneous AP's, but also provide stability and robustness to pacemaker function. Specifically, under

the conditions when the ion channels alone fail to generate a subsequent AP, an internal Ca^{2+} oscillator sends a “healthy” and timely I_{NCX} signal to the membrane, thereby accelerating its DD, thus prompting the membrane to generate the next AP (186).

The most recent variant of this SANC model is a multi-compartment SR model that reproduces individual stochastic LCR's. Varying frequency, size and phase of the LCR's according to experimental data, this model predicts the wide range of chronotropic effect with graded PKA activity, that is, basal and reserve rate regulation (156) (Fig. 12D, triangles; Fig. 14: D and E). Also, the multiple stochastic LCR's in the model account for beat-to-beat cycle length variations, approx. 2% (V.A. Maltsev, unpublished results) which can contribute to variations due to stochastic opening and closing of individual ion channels described previously (187).

10.3. Future SANC modeling requires spontaneous Ca^{2+} release mechanisms

The two above numerical model approaches illustrated that the concept of Ca^{2+} release ignited excitation is indeed operational in primary SANC, based on the existing knowledge on LCR's 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). A future approach includes a theoretical oscillatory spontaneous Ca^{2+} release mechanism. New formulations include the specific mechanisms of the enhanced Ca^{2+} cycling in SANC, due to an enhanced phosphorylation of its Ca^{2+} cycling proteins. Preliminary studies (V.A. Maltsev, unpublished results) have adopted rabbit ventricular myocyte Ca^{2+} release formulations with SR Ca^{2+} -dependent RyR kinetics (188) for a rabbit SANC operation. The current model (157) with the upgraded Ca^{2+} release formulations now predicts theoretically (rather than phenomenologically) the complete cycle of the Ca^{2+} clock including the phase of the spontaneous Ca^{2+} release and Ca^{2+} clock-driven AP's with the rate being tightly regulated by PKA-dependent phosphorylation.

11. Embracing the integration of intracellular and membrane processes manifested by natural pacemaker cells in engineered biological pacemakers

It has long been known that early embryonic cardiac cells are indeed truly pacemakers. As long as approx. 40 years ago, it was noticed that in 1-day-old heart cells, the transmembrane potential tended to oscillate (189). In

7-day chick embryos, when spontaneous AP's are blocked with TTX, heart cell aggregates continue oscillatory pacemaker potentials, which are strongly Ca^{2+} -dependent (190). It was the voltage noise recordings by DeFelice and DeHaan (130, 131) in chick heart cell aggregates that inspired Irisawa in 1978 (94) to suggest a role of Ca^{2+} oscillations in normal cardiac pacemaker function.

There has been a recent, explosive interest in embryonic cardiac myocytes (and stem cell-derived cardiomyocytes) with respect to engineering cardiac tissue, particularly regarding the idea of creating a rather simple “biological pacemaker” for clinical use (191). Approaches taken to date however, have failed to embrace the critical role of intracellular Ca^{2+} cycling in normal pacemaker function (191 – 197).

Ca^{2+} cycling in embryonic cardiomyocytes is now the subject of extensive experimental research. Single cell experiments showed that cardiomyocytes, at a very early developmental stage, indeed possess a membrane-independent Ca^{2+} oscillator that drives spontaneous contractions in these cells (198). This early Ca^{2+} oscillatory activity also evokes membrane depolarizations that trigger I_{CaL} -driven AP's. I_{CaL} is present in the stem cell-derived cardiomyocytes when they first beat, and its density dramatically (approx. 3 times) increases during further differentiation (199). Ca^{2+} cycling proteins (RyR2 and SERCA2a) are expressed very early in cardiomyocyte development (Fig. 15A), and SR Ca^{2+} cycling is crucially important in the developmental regulation of Ca^{2+} transients and contraction in developing cardiomyocytes (200). Knocking out the cardiac type RyR type 2 in mice results in embryonic lethality (201), a result that has later been interpreted to be due to a failure of spontaneous Ca^{2+} releases within embryonic cardiac cells (202). This leads to a marked depression of the obligatory increase in heart rate and cardiac output that support continued cardiac differentiation and growth (202) (Fig. 15: C and D). A crucial regulatory role of RyR type 2-mediated Ca^{2+} releases in the pacemaker rate regulation is indicated by observations that β -AR stimulation is ineffective in RyR type 2 knockout pacemaker cardiomyocytes (200) (Fig. 15B).

In addition to RyR, IP3-dependent Ca^{2+} signaling has recently been found to be critically important for pacemaker activity in early cardiogenesis (203). The underlying spontaneous oscillatory local Ca^{2+} releases have been recently identified by confocal microscopy in developing cardiomyocytes under voltage clamp and in saponin-permeabilized cells (204). Two major types of releases, that is, mediated by both RyR and IP3, receptor were identified, based upon different fine spatio-temporal characteristics, basal Ca^{2+} sensitivity, and

Fig. 15. A: Ca^{2+} cycling proteins are expressed early during cardiomyocyte differentiation from embryonic stem (ES) cells. Shown are RT-PCR analyses of transcripts for type 2 ryanodine receptor (RyR2), juncophilin-2 (JP-2), calsequestrin (CSQ), phospholamban (PLB), SERCA2a, and housekeeping gene β -tubulin at various differentiated stages: ES, undifferentiated ES cells; 2, 5, and 7 days before plating; Day 7 + 3, 7, and 11 days after plating. B: The ISO-stimulated increases in frequency of spontaneous Ca^{2+} transients in cardiomyocytes derived from wild type and RyR-2^{-/-} ES cells at different differentiation stages (early, intermediate, and late). ISO does not increase frequency of spontaneous Ca^{2+} transients at all stages of differentiation of RyR-2^{-/-} cells. C: Beating rate in the RyR-2^{-/-} cardiomyocytes within embryoid bodies and its increase with developmental time is significantly suppressed compared to RyR-2^{+/+} cells. D, E: AP recordings in representative RyR-2^{+/+} and RyR-2^{-/-} cardiomyocytes. Firing rate of RyR-2^{+/+} cell before (middle, 71 b/min) and after (bottom, 26 b/min) ryanodine (10 μ M). The firing rate of RyR-2^{-/-} cardiomyocytes before (middle) and after (bottom) exposure to ryanodine did not change (26 b/min). Panels A and B from ref. 200, C – E from ref. 202.

pharmacological properties (205).

Crucial importance of NCX in coupling intracellular Ca^{2+} to membrane potential rhythms in the embryonic cardiomyocytes (206) and in ES cell-derived cardiomyocytes (207) has been suggested based on I_{NCX} patch clamp measurements. Both studies reported a higher density of NCX and a larger I_{NCX} specifically at the very early stages of cardiac cell differentiation. Also, the importance of NCX in embryonic cardiomyocytes is supported by observation that NCX knockout mice are embryonic lethals, without evidence of the heart ever beating (208). In contrast, it is interesting to note that hearts of mouse embryos lacking HCN4 (the “pacemaker” I_f channel) contract rhythmically, albeit at a reduced (approx. 50%) rate, that is, “a basal heart rate can be sustained without I_f ” (209).

Based on these data, we believe that the pacemaker mechanism of embryonic cardiac pacemakers is not simple as thought before, that is, a mechanism generated by a reciprocal activation of a few voltage-gated membrane ion currents (199, 210). Rather it is a complex

integration of RyR- and IP_3 -based Ca^{2+} oscillators with the membrane clock via I_{NCX} (i.e., similar to adult pacemakers). Thus, it is our opinion that serious consideration of Ca^{2+} -cycling is critical for future bio-engineering of viable, stable/robust, and responsive cellular pacemakers for clinical applications.

12. Summary and conclusions

Concepts regarding mechanisms that underlie the rhythmic heart beat have gone through multiple evolutionary cycles driven by new ideas and experimentation for the last 200 years. However many of these concepts were somewhat “polarized”, highlighting either strictly a subcellular or a membrane-delimited origin of cardiac pacemaker automaticity, for example, Bozler’s 1943 “metabolism” oscillator or the 1962 Noble model, respectively (Fig. 5).

Intracellular Ca^{2+} cycling had been previously studied mainly in ventricular (and atrial) muscle cells. Recent and extensive molecular and Ca^{2+} dynamic studies in

SANC has reunited pacemaker and ventricular cell research along the idea of the crucial role of Ca^{2+} cycling operated by the very same molecules in both cell types (Fig. 1). The Ca^{2+} driven pacemaker mechanism described in our review is not unique to SANC but represents a general mechanism of normal pacemaker function for other cardiac cells, for example, latent atrial subsidiary pacemakers, Purkinje cells (58), AV nodal cells as well as of embryonic and ES cell-derived cardiomyocytes. However, the Ca^{2+} clock in muscle cells and pacemaker cells and its interaction with cell membrane and cell organelles are fundamentally different, as they serve different functions of these cells: EC-coupling and generation of robust rhythmic excitations, respectively. In ventricular cells, intracellular Ca^{2+} cycling is entrained and serves as a high gain chemical amplifier within CICR paradigm. Although CICR is operational in pacemaker cells, too, it mainly appears to serve as a component of the Ca^{2+} clock, resetting SR Ca^{2+} cycling mechanisms, rather than to initiate contraction in the cells (contractile force seems to be a minor SANC function). The Ca^{2+} clock of SANC serves as an internal generator of rhythmic signals subsequently transformed by NCX to electrical signals and greatly amplified by voltage-gated ion channels to effect rhythmic AP's.

Robust evidence indicates that the Ca^{2+} clock does not merely “fine tune” the SANC AP firing rate but are the formal cause for spontaneous AP's and normal automaticity. A high degree of basal PKA-dependent protein phosphorylation permits the generation of basal LCR's in SANC. β -AR stimulation extends the range of PKA-dependent rate control. Furthermore, the crucial role of normal RyR function in mediating the chronotropic response to β -AR stimulation can also be demonstrated in the intact organism, in vivo (156). 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 LCR and membrane clocks. Vagal stimulation of cholinergic receptors predominates in the basal state, keeping the LCR clock in check. In response to a demand for higher heart rates, stimulation of cholinergic receptors wanes, and sympathetic stimulation of β -AR waxes. At the maximum heart rate, β -AR activation of PKA-dependent phosphorylation is maximal, and the LCR Clock runs at full speed, to effect the maximum heart rate.

In conclusion, the true cardiac pacemaker mechanism is complex and includes a balanced integration of both intracellular and membrane delimited processes. This type of integration seems to be imperative for the bio-engineering of robust biological pacemakers. The emerging evidence for the crucial role of Ca^{2+} and

rhythmic spontaneous Ca^{2+} releases in the initiation and regulation of normal automaticity, in our opinion, provides the key that reunites pacemaker and ventricular cell research. 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: Calcium cycling into and from internal stores by proteins common to both ventricular myocytes and pacemaker cells regulate the duty cycle of both cell types. Thus, a general theory of cardiac inotropy and chronotropy emerges!

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