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Review

Mechanisms underlying the cardiac pacemaker: the role of SK4 calcium-activated potassium channels

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The proper expression and function of the cardiac pacemaker is a critical feature of heart physiology. The sinoatrial node (SAN) in human right atrium generates an electrical stimulation approximately 70 times per minute, which propagates from a conductive network to the myocardium leading to chamber contractions during the systoles. Although the SAN and other nodal conductive structures were identified more than a century ago, the mechanisms involved in the generation of cardiac automaticity remain highly debated. In this short review, we survey the current data related to the development of the human cardiac conduction system and the various mechanisms that have been proposed to underlie the pacemaker activity. We also present the human embryonic stem cell-derived cardiomyocyte system, which is used as a model for studying the pacemaker. Finally, we describe our latest characterization of the previously unrecognized role of the SK4 Ca^{2+}-activated K^+ channel conductance in pacemaker cells. By exquisitely balancing the inward currents during the diastolic depolarization, the SK4 channels appear to play a crucial role in human cardiac automaticity.

Keywords: cardiac pacemaker; sinoatrial node; SK4 K^+ channel; Ca^{2+} clock model; voltage clock model

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Introduction

Normal cardiac function depends on the adequate timing of excitation and contraction in the various regions of the heart and on an appropriate pacemaker rate. This complex task is implemented by the highly specialized electrical properties of the various elements of the heart system, including the sino-atrial node (SAN), atria, atrioventricular node (AVN), His-Purkinje conducting system, and ventricles. Additionally, the different regions of the heart are endowed with various combinations of ion channels, pumps and exchangers yielding distinct action potential (AP) waveforms and durations.

Although all cardiomyocytes are initially endowed with pacemaker activity in the early embryonic stages, most cardiac cells eventually differentiate into the working myocardium, which lacks pacemaker properties. Only a small population of embryonic cardiomyocytes form SAN, AVN and the bundle of His[1]. In this short review, we survey the development of the human cardiac conduction system and the various mechanisms that have been proposed to underlie the pacemaker activity. We also present the human embryonic stem cell-derived cardiomyocyte system, which is used as a model for studying the pacemaker, and the most recent characterization of the previously unrecognized role of the SK4 Ca^{2+}-activated K^+ channel conductance in pacemaker cells.

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Development of the human cardiac conduction system

The human heart becomes the first functional organ at the beginning of the 3rd week of development. After 21 days of embryogenesis, a human embryo measures less than 5 mm, and the primordial heart is a "cardiac tube" that is connected to the circulation system through the inflow and outflow tracts at the entrance and at the exit of the nascent organ, respectively (Figure 1A). The primitive cells, which derive from the cardiac mesoderm, have not yet developed sarcomeres and endoplasmic reticulum. However, the primitive cells exhibit the molecular signatures of "slow conduction", such as the expression of the T-box transcription factor Tbx3, the hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4)[2, 3], the L- and T-type voltage-gated calcium channels, and connexin (Cx) 45. Connexin 30.2 is also specifically expressed in the primitive mouse heart[4], but its human analog, Cx 31.9, is not detectable[5]. Taken together, these characteristics confer an asthenic peristaltic myogenic oscillatory automaticity to the single chamber organ, which is observed as a sinusoidal signal in an ECG (Figure 1A)[6]. Importantly, all of the cells exhibit an intrinsic spontaneous pacemaker activity, but a specific

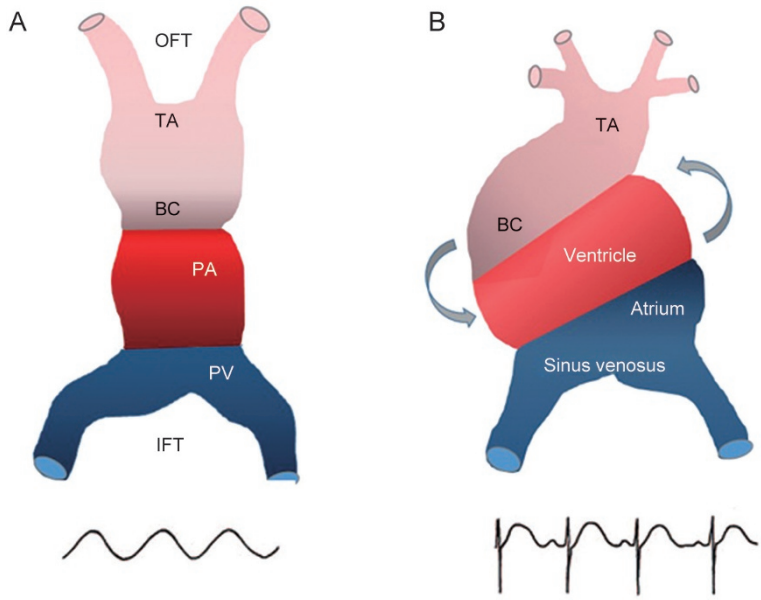

Figure 1. Schematic overview of heart development and the associated ECG. (A) At 21 days of embryogenesis, the cardiac tube is connected to the inflow (IFT) and outflow tracts (OFT) and generates a primitive activity and a sinusoidal ECG. TA, truncus arteriosus; BC, bulbus cordis; PA, primitive atrium; PV, primitive ventricle. (B) At 24 days of embryogenesis, there is expansion of the chambers and the appearance of an "adult-like ECG". The sinus venosus, atrium and ventricle appear.

subpopulation of cells that are localized in the posterior part of the tube contracts faster and imposes a higher rate, which leads to unidirectional circulation from the venous pole (influx tract) to the arterial pole (outflow tract)[7].

The necessary structural and physiological adaptations of the primitive heart occur with the growth of the embryo and the sophistication of the circulation system. The proliferation of the atrial and ventricular balloons, which are located dorsally and ventrally to the cardiac tube axis, respectively, give rise to the following distinct areas: the sinus venosus, the atrium, the atrioventricular canal, the left ventricle, the right ventricle and the aortic sac (Figure 1B). Throughout the "looping process" during the formation of the chambers at the end of the first month of development, the heart folds and adopts an "S-shape"[8]. These structural modifications are accompanied by changes in the electrical properties of the organ, causing heterogeneity in the conduction system[9, 10]. The sinus venosus and the atrioventricular canal exhibit the same low-velocity pattern as the primitive cells of the cardiac tube, whereas the newly synthetized cells (chamber areas) display the characteristics of a "high-velocity/high pressure" system. Histologically, this proliferative "pre-chamber myocardium" contains well-organized sarcomeres and developed endoplasmic reticulum. Molecularly, the extinction of Tbx3 leads to the appearance of "high conductance" proteins[11-13], such as the connexins Cx40 and Cx43[14], and the replacement of the L- and T-type Ca^{2+} channels by the fast voltage-activated sodium channel Scn5a (Nav1.5)[15]. HCN4 expression is also strongly decreased in the working myocardium, as this tissue loses its intrinsic pacemaker activity[16-18]. The ventricular conduction is provided by the trabecular myocardium from the dorsal part of atrioventricular canal, which expresses the fast-conducting connexins[19, 20]. The specific characteristics of the AV canal are responsible for the "AV delay", an electrical coupling between the slow and fast-conducting segments. This establishment of a two-speed conducting system at the 25th day of development is a critical feature for the proper functioning of the heart[21], as it guarantees that the ventricular segment will not contract before the contraction of the upstream portions of the heart ends. In the sinus venosus, HCN4 and Tbx3 expression are progressively restricted to a specific area, the "sinus node primordium" or "primary sinoatrial node"[22]; the other cells from this structure (right and left sinus horns) eventually lose their spontaneous activity and become working atrial myocytes during the "atrialization process". This primary sinoatrial node then grows and forms a "head" that extends into the right sinus horn[23]. Small networks of cells, the internodal tracts, connect the SAN to the remodeled AV area and send projections into the right and left atria (Bachmann's bundle)[17, 24]. Another SAN can be observed in the left part of the sinus horn, but this transient structure usually degenerates rapidly[25]. A large portion of the atrioventricular canal gradually disappears by differentiation into the working myocardium or apoptosis. Only a small continuum of the $Tbx3^+$/ $Cx45^+$/$HCN4^+$ primitive myocardium remains active in the conductive system. At approximatively the 5th week of embryogenesis, these cells give rise to the "atrioventricular node" (AVN), the right and left atrioventricular ring bundles. These ring bundles surround the forming atrioventricular valves[26]. The left bundle, which is already observed in the young embryonic heart, is localized close to the mitral valve. The right bundle, which develops in fetal and neonatal stages, forms around the tricus-

pid valve. A fibrous structure then separates the atria from the ventricles, preventing any electrical coupling and potential arrhythmias, and the AV node remains as the only myocardial connection point between the atria and the ventricles. The AV bundle emerges at the crest of the forming interventricular septum. This structure displays increasing levels of high conduction proteins (CX40) while sustaining the levels of primitive marker Tbx3. As the AV canal shrinks, another fast conduction system is required to supply the growing ventricles[1]. The "peripheral ventricular conduction system" is the last element of the conduction system to differentiate. In contrast to the other conductive structures, it does not arise from the $Tbx3^+$ myogenic precursors of the cardiac tube. Although the ventricles develop, two different synthetized myocardium subpopulations appear: a "compact working component" and a "trabecular component". The trabecular myocardium is the origin of the peripheral ventricular conduction system. These cells still display spontaneous pacemaker activity, express HCN4, and exhibit a "high-velocity Cx45/Scn5a phenotype". The left and right His branches emerge during the interventricular septation, when the trabecular myocytes penetrate inside the compact components of the septum[27]. With the thickening of the ventricle lateral and ventral walls, the trabecular myocytes develop an additional structure, the subendocardial Purkinje network[21, 28], which notably develops in response to an endothelial paracrine effect during the vascularization of the ventricles.

In the mature human heart and under physiological situations, the pacemaker activity is generated in the SAN, the dominant structure of the conduction system, which is localized in the top region of the right atrium close to the superior vena cava (Figure 2). The cyclic and slow spontaneous depolarization spreads through the internodal tract and Bachmann's bundle to reach the AV node at the base of the right atrium. This atrial contraction or "atrial systole", which is visible as a "P-wave" in the ECG, pushes the blood into the ventricles (Figure 2). The low-velocity characteristics of the AV node are crucial for a complete, active ventricular filling. The AV delay or "P-Q interval" is observable in the ECG as a segment of the isoelectric line following the P-wave. The electric impulse then reaches the His bundle and the fast-conducting components of the conduction system. Depolarization occurs rapidly, splitting into the two His branches of the interventricular septum and spreading through the Purkinje network inside the myocardium, leading to the "ventricular systole", known as the contraction of the ventricles from the apex to base, and ejection of the blood into the systemic or pulmonary circulation. This depolarization occurs from the endocardium through the epicardium and can be observed as the "QRS complex" in the ECG (Figure 2). Finally, the ventricles repolarize. This is the "ventricular diastole" or "T-wave" in the ECG, where the ventricles relax and then progressively start

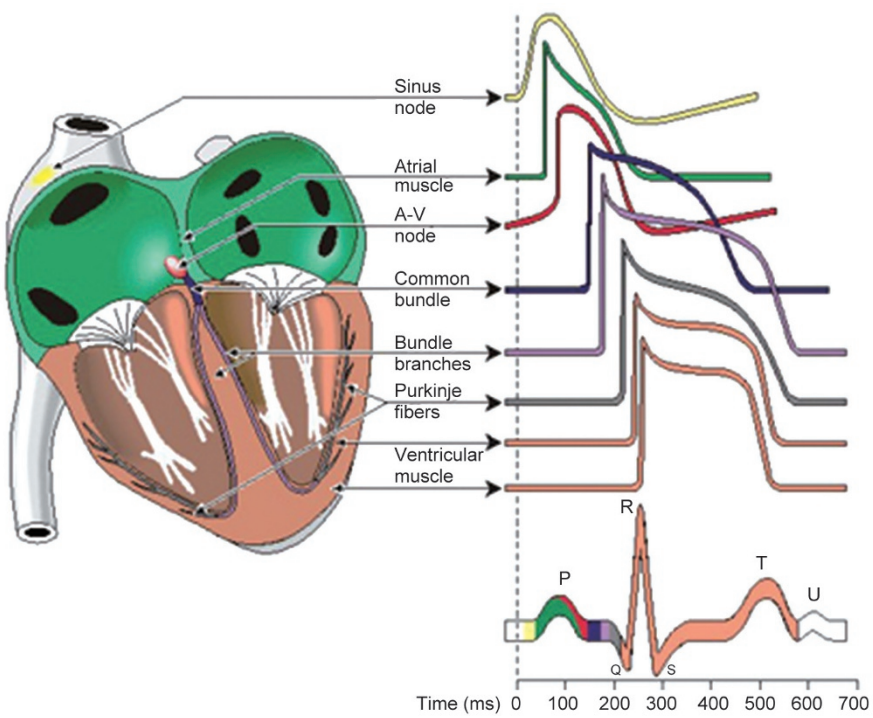

Figure 2. The mature conduction system in the adult heart and associated ECG. Representative cartoon of the organized pacemaker tissue in the mature human heart. The different regions of the heart and the conduction system are shown, including the sinus node, the atrial muscle, the A-V node, the common bundle, the bundle branches, the Purkinje fibers and the ventricular muscle. The typical action potentials for each region and their respective contribution to the ECG are shown. A typical trace of an ECG shows the "P-wave", the "P-Q interval" (isoelectric line), the "QRS complex", the "T-wave" and the "U-wave".

their passive filling process. The "U-wave" is thought to represent the repolarization of the papillary muscles or Purkinje fibers.

Although the AV node, AV bundle, His and Purkinje cells display spontaneous electric activities when isolated and recorded with the patch clamp technique, their intrinsic firing rate is lower than the SAN cells (30–40 depolarizations per minute in the His/Purkinje cells, 40–50 in the AVN or AV bundle, and approximately 60–75 in the SAN). This "SAN overdrive suppression" on the other elements of the conductive network explains its leading role in driving the pacemaker. Therefore, these structures are confined to a propagative function, even if they can act as a "pacemaker backup" in the case of SAN failure. However, due to their intrinsic electrical properties, they can also become potential arrhythmogenic sources under pathophysiological situations.

Proposed mechanisms accounting for the cardiac pacemaker activity

Jan Evangelista PURKINJE identified a ventricular network in 1839, Whilhem HIS JR observed a bundle within the interventricular septum in 1893, and Sunao TAWARA identified the AV node in 1906. The SAN was anatomically discovered by Arthur KEITH and Martin FLACK in 1907, although a pacemaker structure in the right atrium was proposed by Walter GASKELL much earlier in 1880. While the anatomical structures of the conduction system were identified more than 100 years ago, the molecular mechanisms underlying the cardiac automaticity remain controversial.

The pacemaker activity of a cell can be defined as its ability to spontaneously and cyclically generate an electric signal. To achieve this, the membrane potential of the SAN cell needs to slowly depolarize to a threshold, which is a specific voltage required for the generation of an AP. This depolarization, called "Diastolic Depolarization" (DD) or "pacemaker depolarization", results from a small net inward current flowing across the cell membrane and is the key feature of cardiac automaticity. DD occurs during diastole at the end of an action potential and is responsible for triggering the next action potential. Without diastolic depolarization, the heart would simply be unable to beat spontaneously. A crucial requirement for rhythmic automaticity is the existence of inward currents at diastolic potentials and a subtle dynamic integration of the sarcolemmal ion channels, transporters and Ca^{2+} cycling proteins[29].

Membrane or voltage clock model

This model involves a combination of different ion channel activities during diastolic depolarization. One of the most important candidates is the "funny current" or "I_f" (I_h in neurons) that was discovered in 1979 and found to be responsible for heart rate acceleration under noradrenaline stimulation[30, 31]. Initially mistakenly described as a "potassic" current[32, 33], this "pacemaker current" was actually a non-specific inward cationic current produced by the hyperpolarization-activated and cyclic nucleotide-gated channels (HCN channels), which structurally resemble the K^+ channel superfamily[34]. Despite the presence of a potassium selectivity filter motif (GYG) and a global structure that resembles the potassium channels, HCN channels are also permeable to Na^+ [35] and, to a much lesser extent, to Ca^{2+} ions[36]. At physiological voltages, the Na^+ influx via the HCN channels is larger than the K^+ outflow. Functionally, the I_f current is distinguished by its unique electrophysiological properties. Whereas all of the other existing voltage-gated cationic currents are activated by depolarization, the HCN channels open in response to membrane hyperpolarization. This difference explains the reason why the atypical current was also called the "funny current". Physiologically, at the end of an action potential, the membrane hyperpolarization activates the HCN channels, which slowly generate an inward depolarizing current until it reaches the threshold and subsequently generates a new action potential (Figure 3, left panel).

Four isoforms of the HCN channels are expressed in mammals, and their expression levels vary considerably depending on the animal model[34, 37]. It is generally accepted that the HCN4 subtype is the most abundantly expressed isoform in the cardiac conductive tissue, with predominance in the human, rabbit and rodent SAN[38]. In addition to HCN4 and according to their transcript levels in SAN, HCN2 is expressed in mouse and HCN1 is expressed in rabbit[39]. HCN3 was recently observed in rodent ventricular myocytes[40]. As HCN3 has a lower range of activation in working cardiomyocytes, the I_f current density is very small in those type of cells, which may be why it is considered as a "background current", which is in contrast to its role in SAN[41]. The funny current is regulated by cAMP, which directly binds to a "cyclic nucleotide-binding domain" at the C-terminus of the channel. In response to sympathetic activity, adenylate cyclase increases intracellular cAMP levels, which leads to a "rightward-shift" of the I_f activation curve, and, subsequently, a larger current at the same voltage. This modulation explains the stronger diastolic depolarization slope (DD slope) and the increase in pacing rhythm in response to adrenaline. By contrast, acetylcholine decreases the cAMP levels in the SAN, leading to a smaller current at the same voltage (left-shift of the activation curve), a weaker DD slope and bradycardia[42]. The β accessory subunit MiRP1 changes the activation kinetics of the current in opposite ways depending on the HCN isoform. Although it has been reported to increase the activation kinetics and the currents of HCN1 and 2[43, 44], it decreases the activation kinetics but increases the I_f current when it is associated with HCN4[45]. Other HCN regulatory mechanism have been described, including phosphatidylinositol 4,5-bisphosphate (PIP_2) modulation[46] or the direct actions of kinases[47] and phosphatases[48, 49]. Loss of function of HCN4 due to mutations or nonspecific pharmacological blockade leads to sick sinus syndrome, which is a general inability of the SAN to impose its sinusal rhythm on the conducting system. In most cases, this disease is the manifestation of an idiopathic degeneration of the SAN leading to arrhythmia, such as sinus bradycardia, alternating periods of tachycardia and bradycardia[50] (tachy-

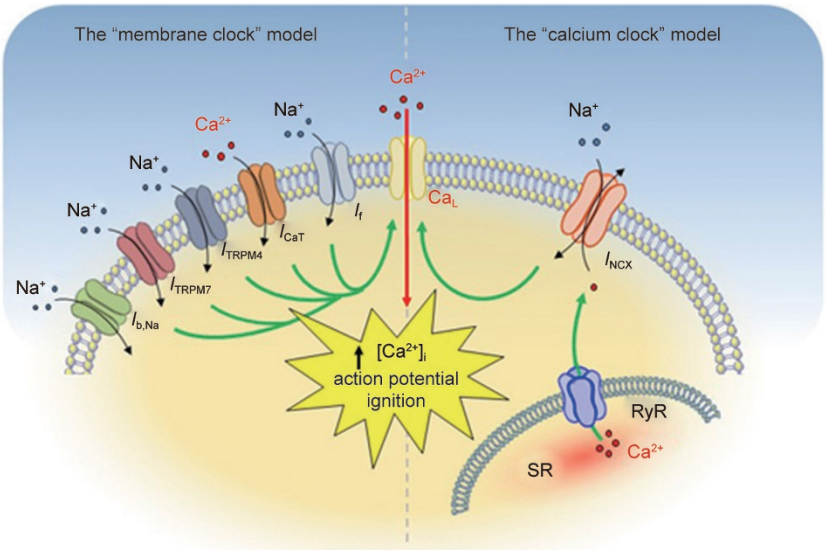

Figure 3. The "membrane clock" model. This model is a general, integrative model that includes the different channels or channel-receptors that are involved in this process. The I_f, the sustained $I_{b,Na}$, I_{CaT} (Cav3.1 and Cav3.2), I_{TRPM4} and I_{TRPM7} inward currents are all good candidates that may be responsible for the diastolic depolarization, alone or in combination. The "calcium clock" model (adapted from EG Lakatta, Ref [85]). Diastolic spontaneous calcium release increases the cytoplasmic calcium concentrations and activates NCX-1 in the forward mode. The inward I_{NCX} current depolarizes the membrane until the threshold is achieved.

brady episodes) or, in the worst cases, to sinus arrest. Animal models of total or partial HCN knockout yielded varying results. A specific and unconditional atrial suppression of HCN4 in the knockout mice leads to *in utero* death without histological abnormalities, whereas electrophysiological studies of the isolated cells from these mouse embryos showed a reduced level of spontaneous pacemaker activity, despite the 75%–90% decrease in the I_f current density[51]. The same study observed that cAMP regulation was impaired in the cardiomyocytes from HCN4$^{-/-}$ mice, whereas another study presented opposite results with a HCN4 conditional knockout in adult mice[52]. Taken together, these results indicate that the funny current is not the only component of the pacemaker diastolic slope (Figure 3, left panel).

In 1990, Denyer and Brown performed electrophysiological studies on rabbit SAN cells. Although they used cesium at a concentration of, which nearly completely blocked the I_f, the spontaneous firing rate of the cells was reduced but not inhibited. After confirming that the cesium blockade of the I_f did not induce changes in the other inward or outward currents, they conclude that a sustained, previously unidentified current is present in the SAN cells[53]. The conductance was better characterized in a subsequent study as a poorly selective inward cationic current[54] even though it exhibits a stronger permeability to sodium[55]. Interestingly, this $I_{b,Na}$ (sodium background) current is insensitive to high concentrations of TTX but can be modulated by the calcium channel blocker nicardipine[56] or the agonist Bay K644. Surprisingly, this sustained current is also regulated by beta agonists or by the parasympathetic system, similar to I_f. Although the mechanism has not yet been described, isoproterenol increases the current whereas acetylcholine produces the opposite effect[57]. $I_{b,Na}$ has been reported in rabbit, rat and guinea pig SAN cells[58]. With an activation threshold at approximately -60/-70 mV and a maximum current of -50 mV, this channel can easily contribute to the DD slope in synergy with I_f (Figure 3, left panel).

Two different subfamilies of voltage-gated calcium channels are expressed in the heart pacemaker tissue[59-61], the high-threshold "L-type" ($Ca_v1.2$, $Ca_v1.3$) and the low-threshold "T-type" channels (Ca_v 3.1, Ca_v 3.2), which are distinguished by their different electrophysiological and pharmacological properties. The high-threshold L-type channels begin to be activated at –30 mV, whereas the T-type channels are activated at more hyperpolarizing voltages (approximately –60 mV). The T-type isoforms $Ca_v3.1$ and $Ca_v3.2$ appear at an early stage of cardiogenesis and are mainly localized in the AV canal. In the healthy adult mammalian heart, they are not expressed in the working cardiomyocytes but have been identified in the SAN, AVN and Purkinje fibers[62]. In the SAN, the levels of the $Ca_v3.1$ transcript are higher than $Ca_v3.2$[63]. This restriction of the T-type channels to the conductive system tends to indicate a hypothetical specific involvement in the pacemaker activity. In fact, $Ca_v3.1$ knockout mice display arrhythmias, such as bradycardia, and an AVN delay in ECG. Spontaneous action potentials recorded from isolated SAN or AVN cells from the $Ca_v3.1$ knockout mice have a shallower DD slope and a lower instantaneous firing rate than wild-type cells[64]. Given that wild-type cells are activated by hyperpolarizing membrane voltages, the inward currents of these channels can contribute to the pacemaker slope (Figure 3, left panel).

L-type Ca^{2+} channels have been described as having a crucial role in the excitation-contraction coupling (E–C) as they

are expressed at high levels in skeletal muscle and the working myocardium. In fact, their sustained activity in phase 2 of an AP (plateau) is necessary for a proper interaction between the sarcomeric molecules and a contraction of the myocytes. $Ca_v1.2$ and $Ca_v1.3$ are expressed in the SAN pacemaker cells[61], but unlike their role in the working myocytes, these currents are essential for generating the action potential upstroke of the "low-velocity" pacemaker cells. $Ca_v1.2$ is a ubiquitous current that is expressed equally in all human heart regions, whereas $Ca_v1.3$ was expressed more specifically in the SAN and the human AVN[65]. Pharmacologically, there are currently no reagents available to discriminate between the two distinct currents, but they can be distinguished based on their voltage-dependent activation. $Ca_v1.2$ channels exhibit slower activation kinetics, which occurs at more depolarizing membrane potentials than for $Ca_v1.3$[66, 67]. ECG performed on $Ca_v1.3^{-/-}$ null mutant mice revealed bradycardia, SAN dysfunction and an AV block[68]. APs that were spontaneously recorded from isolated SAN or AVN cells also showed a decrease in the pacing rate and the DD slope, pointing to the important role of these channels in the pacemaker function[65, 67].

Recently, different transient receptor potential (TRPM) channels have also been described as having a role in this physiological process. TRPM4, a non-selective, calcium-activated cation channel was characterized in the rodent SAN[69]. This sodium and potassium inward current is positively regulated by $[Ca^{2+}]_i$ and negatively regulated by ATP. These channels have also been reported in the SAN from other species[70, 71]. Pharmacological inhibition or full suppression in knockout mice leads to a decrease in the AP rate (Figure 3, left panel). Some other channels have also been suggested to be involved in the pacemaker activity but more as regulatory elements than main components of the DD slope. This is the case for TRPM7, a divalent cation-permeant channel kinase that is expressed in various tissues, including the heart. Although conditional deletion in late-stage developing embryos does not disturb cardiac development, intermediate-stage or early-stage knockout leads to a decrease in HCN4 expression in the primitive heart, congestive heart failure or *in utero* death, respectively[72]. Restricted TRPM7 deletion in the mouse SAN disrupts cardiac automaticity *in vivo*, leading to sinus pauses and an AV block. Molecularly, TRPM7 knockdown alters HCN4 expression, leading to its downregulation and subsequent changes in automaticity[73].

Inward-rectifier potassium currents, such as I_{KACh} or I_{KATP}, which are expressed in the SAN, can also modulate the DD slope. The I_{KACh} channels open in response to acetylcholine binding to the muscarinic receptor in the SA. The consecutive hyperpolarizations that are generated lead to a reduction in the pacing rate[74]. Interestingly, cholinergic modulation also affects I_f, but with a different affinity. High levels of acetylcholine are required to activate I_{KACh}, whereas nanomolar concentrations are sufficient to regulate the funny current[75]. I_{KATP} is activated in response to a reduction of the cytoplasmic ATP levels[76, 77]; similar to I_{KACh}, its outward current hyperpolarizes the membrane and decreases the pacing rate.

Calcium clock model

In 1972, Fabiato and colleagues[78] observed that cardiomyocytes from which the membrane has been removed still exhibited spontaneous contractions. Calcium is known to play a central role in the E-C coupling in "working" cardiomyocytes. In fact, during this physiological process, the depolarization of the sarcolemma generates an inward calcium current through the activation of the L-type Ca^{2+} channels, which is responsible for triggering a "calcium-induced calcium release" (CICR), a synchronous calcic wave, from the ryanodine (RyR) and IP_3 receptors of the sarcoplasmic reticulum (SR). The subsequent $[Ca^{2+}]_i$ increase is a key feature of a proper actin-myosin interaction and cardiomyocyte contraction [79-81]. In addition to the calcic wave, which results from a synchronous release through the SR receptors, spontaneous local calcium sparks have been observed in working myocardium[82, 83] and Purkinje fiber cells[84]. These spontaneous Ca^{2+} oscillations occur locally in a submembrane region of the cell and propagate rapidly. These phenomena were initially thought to be only associated with cardiac arrhythmias[85] until they were also reported in atrial pacemaker cells[86]. In fact, with the help of imaging techniques, Ca^{2+} chelating fluorophores and electrophysiology, Ca^{2+} transients were visualized during the DD slope and preceded the large entry of calcium through the calcium channels[87]. Alternative pharmacological approaches with SR blockers revealed modifications in the rate and the shape of the AP from guinea pig sinoatrial cells[88]. Evidence for a functional interaction between the ryanodine receptor-mediated Ca^{2+} release and the cardiac isoform of the NCX-1 Na^+-Ca^{2+} exchanger[89-91] gave rise to an alternative theory, "the calcium clock", in which diastolic spontaneous Ca^{2+} release activates the Na^+-Ca^{2+} NCX-1 exchanger in its forward mode. In these conditions, the net electrogenic inward current resulting from the entrance of 3 Na^+ and the extrusion of 1 Ca^{2+} depolarizes the membrane of the cell toward the threshold and generates an action potential[92, 93] (Figure 3, right panel).

RyR2 and NCX-1 can be modulated by adrenergic stimulation. Indirect evidence showed that SAN cells displayed stronger and longer calcium sparks in response to isoproterenol and higher I_{NCX} currents[89, 94, 95]. Molecularly, the regulation mechanism occurs through direct cAMP/PKA-mediated regulation of RyR2[94, 96] and eventually I_{NCX}, although this latter assumption is still being debated[97]. Conversely, as a homeostatic feedback mechanism, the regulation by Ca^{2+}-calmodulin kinase II (CAMKII) decreases the Ca^{2+} sparks to stabilize them[98]. Mutations in RyR2 can lead to a cytoplasmic calcium leak and severe arrhythmias, such as the catecholaminergic polymorphic ventricular tachycardia CPVT1. Hyperphosphorylation of this receptor is also associated with heart failure[99]. An inducible general RyR2 knockout in mouse heart produces a reduced heart rate and severe arrhythmias in the ECG[100]. Similar to HCN, animal models with a total or targeted NCX-1 knockout gave inconsistent results. A total suppression of NCX-1 leads to embryonic lethality due to the absence of the spontaneous beating activity of the heart, despite the normal structural development and the existence

of I_f currents. Paradoxically, despite the complete sarcomeric disorganization and the absence of spontaneous contraction, the NCX-1$^{-/-}$ knockout in the ventricular and the SAN cells still has intact calcium transients, but they are uncoupled from the sarcolemma[101, 102]. An inducible KO in the SAN from adult rodents is associated with a progressive diminution of the heartbeat, a disturbed pacemaker activity and a decrease in the amplitude of the spontaneous Ca^{2+} sparks[103]. However, other studies reached different conclusions. Another study with a knockout in SAN cells indicated that the cells still displayed a normal basal rhythm but did not respond to sympathetic or parasympathetic regulation[104]. Finally, pharmacological approaches using high concentrations of ryanodine on the entire SAN from wild-type rabbit only moderately decreased its pacing rate[105].

Taken together, the membrane clock and calcium clock models are still highly debated[106] and raise many counterpoints and paradoxes. New theories that integrate both models are emerging[107-109], but it remains challenging to provide a clear answer to the simple question that was asked more than a century ago.

Human embryonic stem cell-derived cardiomyocytes: a model for studying the cardiac pacemaker

For evident bioethical reasons, most studies that investigate the mechanisms underlying the cardiac pacemaker are performed on SAN cells from animals, mostly mice and rabbits. However, these species display significant differences in the values and fluctuations of their basal heart rates compared to humans (450–750 beat/min in mouse, 180–350 beat/min in rabbit, and 60–100 beat/min in human). These variations result from notable interspecies differences in the dynamics and contributions of the calcium handling proteins, in the density of each current, and their regulation. Very few studies, however, have reported data from single cells that were dissociated from an excised human SAN[42, 110]. Each of the studies was performed with isolated cells from a single SAN, but it was possible to compare a few electrophysiological parameters with rabbit cells. The action potentials recorded from human SAN cells are longer (higher action potential duration) with a weaker pacemaker slope, upstroke and firing rate than rabbit cells. The strong variations in the AP profile, and likely in the involvement of each molecular player, underline the fact that animals are a model and one should consider this point before extrapolating the results or a general pacemaker theory from one species to another.

It would be intuitive to use human cells to investigate the process involved in humans. In 1998, Thomson and Itskovitz derived cell lines from human blastocysts[111]. Similar to the studies of mice, the inner cell mass of human embryos was propagated and expanded, giving rise to different human embryonic stem cell (hES) lines. These pluripotent cells have the intrinsic ability for self-renewal and differentiation into the three embryonic germ layers (ectoderm, endoderm, and mesoderm). Following suspension culture, the hES colonies adopt spherical morphologies, or "embryoid bodies" (EBs), which mimic the embryonic shape, and they initiate a regional differentiation process into all type of cells, including spontaneous beating clusters[112]. Isolated cells from these clusters generate action potentials with diverse shapes, calcium transients and functional sarcomeres[113, 114]. Cardiomyocytes derived from human embryonic stem cells (hESC-CMs) are considered to be a very promising therapeutic tool in cardiovascular research, as they have considerable potential for cell transplantation and tissue regeneration[115–117]. In the last decade, many studies have been published to better characterize these cells[118, 119], to improve the production protocols[120–122] or to obtain specific, pure subpopulations[123–125]. In addition, because these cells beat spontaneously, analogous to the cardiac cells of the primitive heart, it is assumed that they can be used as a relevant model to investigate the molecular mechanisms underlying the human cardiac pacemaker activity.

The calcium-activated potassium channel family

The calcium-activated channels exhibit the characteristics of a transducer between the ubiquitous cytoplasmic calcium signals and the electrical variations of the cell membrane. Among the diverse types of channels that exhibit these properties, the calcium-activated potassium channel family (KCa) is heterogeneous. According to their unitary conductance, they have been classified into three subfamilies: BK, IK and SK, which are "big", "intermediate" and "small" conductance, respectively[126].

The BK channels ($K_{Ca}1.1$), or "large conductance Ca^{2+}-activated potassium channels", were first identified in 1979 by Heyer and Lux in the pacemaker neurons of the snail[127] and cloned 15 years later[128]. This subfamily of channels differs from the IK and SK channels in their structure and biophysical properties. In addition to the well-known six transmembrane domains present in each subunit of voltage-gated potassium channels, a S0 membrane segment in the N-terminus and a cytoplasmic hydrophobic C-terminus containing a "calcium bowl" extend the α-subunit. Different β-subunits have been reported to be associated with the main structure of the protein[129–131]. BK channels exhibit a large unitary conductance (≈ 250 pS) and are modulated by increased cytoplasmic calcium concentrations and membrane depolarization[126]; they are abundant in the central nervous system[132], smooth muscle cells[133], and leucocytes and have been recently identified in the mouse sinoatrial node[134, 135]. The protective roles of BK channels, such as spike frequency adaptation, explain their increased expression in excitable cells. Pharmacologically, they are sensitive to charybdotoxin, paxilline and iberiotoxin.

The small conductance calcium-activated potassium channels SK1 ($K_{Ca}2.1$), SK2 (K_{Ca} 2.2) and SK3 (K_{Ca} 2.3) are encoded by three different genes, KCNN1, KCNN2 and KCNN3, respectively. These three channels, which were first cloned from rat and human brain by Köhler *et al* in 1996, exhibit a high sequence homology (60%)[136]. Structurally, they are very similar to the voltage-gated potassium channel superfamily. The α-subunits form a tetramer, each comprising six transmembrane segments (S1 to S6) and cytoplasmic amino and car-

boxyl termini. The S5-P-loop-S6 segments constitute the pore and the potassium selectivity filter, whereas the S4 transmembrane domain contains fewer gating charges than the voltage-gated K^+ channels with only two positively charged residues compared with the 4–5 charges in classical voltage-gated channels[136]. These channels do not have an EF-hand domain motif and their activity is only calcium-dependent. The submicromolar intracellular calcium modulation is explained by the presence of a calmodulin binding site (CMBD) at the C terminus[137,138], which, upon calmodulin interaction, leads to conformational changes and channel opening. All of the SK channels exhibit similar steady-state activation curves for Ca^{2+} (half activation approximately 300–700 nmol/L)[136, 137, 139], which is a relatively low affinity. Electrically, SK channels contrast with Ohmic currents and display a strong inward rectification at positive voltages. The mechanism is still not clear but might be explained by an intracellular Mg^{2+} or Ca^{2+} voltage-dependent block[140]. The three subtypes differ in their tissue expression patterns and their pharmacological sensitivities to the bee venom toxin apamin. In the central nervous system, SK1 and SK2 are primarily expressed in the neocortex and hippocampal regions whereas SK3 is localized in more primitive areas, such as the basal ganglia or the thalamus[141]. They mediate the afterhyperpolarization, which ends the action potential[142]. In the periphery, the SK channels are expressed in T-lymphocytes and atrial cells and play an important role in atrial repolarization[143-148]. SK1 channels are resistant to apamin[136], and their unitary conductance varies from 11 to 26 pS depending on the experimental conditions[149-151]. They can associate with SK2 to form heterotetrameric channels. SK2 channels are highly sensitive to apamin, and their unitary conductance has been reported to vary from 10 to 20 pS [136,139,140]. SK3 channels have a moderate affinity to apamin.

The SK4 intermediate calcium-activated channel ($K_{Ca}3.1$) is encoded by the gene KCNN4, which is localized in the q13.2 region of human chromosome 19[152]. Historically, this channel was discovered by Gardos in 1958, when he noted a correlation between the potassium outflow from erythrocytes and the intracellular EDTA/calcium competition[153]. Four decades later, it was cloned and biophysically characterized[154,155]. Although the main channel is a 428 amino acid protein, different mRNA transcripts have been reported (2.6 and 3.2 kb), suggesting that there are different splice variants. Structurally, SK4 is also very similar to the canonical voltage-gated potassium channel superfamily, although it exhibits low homology (40%) with the other SK subfamily members (Figure 4). Similar to the small calcium-activated channels, SK4 is only modulated by calcium through a calmodulin binding site in its C terminal region[156,157] (Figure 4). In addition to the Ca^{2+}-CaM conformational changes, which are necessary for channel opening, calmodulin itself regulates the assembly and trafficking of the protein to the cell membrane[158]. $K_{Ca}3.1$ also has potential PKA and PKC phosphorylation sites. PKA and cAMP activate the channel[159], in addition to an independent C-terminal ATP-dependent phosphorylation[160, 161]. SK4 is strongly expressed in erythrocytes, placenta, lung, prostate, bladder, thymus, and smooth muscle cells. However, it is almost completely absent in the brain[154,155], although a recent report has demonstrated that SK4 channels are expressed in the nodes of Ranvier of cerebellar Purkinje neurons[162]. Interestingly, until recently, SK4 channels were not detected in the heart[154, 155]. Electrically, its single conductance varies from 10 to 42 pS[163] and also exhibits the same inward rectification as the other SK channels. However, SK4 differs in its higher affinity to intracellular Ca^{2+} (half activation at 95 nmol/L free Ca^{2+}), which confers a functional role to the channel at physiological, basal intracellular $[Ca^{2+}]_i$ concentrations (approximately 100 nmol/L). Pharmacologically, SK4 channels are insensitive to apamin but are blocked by the scorpion toxin charybdotoxin and by different drugs, such as clotrimazole and, more recently, TRAM-34[164].

The "membrane clock" and "calcium clock" in human embryonic stem cell-derived cardiomyocytes (hESC-CMs)

In stem cell research, a growing number of studies have been published the last decade focusing on cardiac regeneration[117, 165] and on the pacemaker mechanism[166–172]. As hESC-CMs usually start to beat spontaneously between the 9th and 12th day after differentiation begins, we focused our work on young cells (d11 to 21), which parallels the early beating cells of the cardiac tube[173]. By alternating the current and voltage clamp configurations of the patch clamp technique with a pharmacological approach on the same cell, we found that 32% of the hESC-CM population exhibited a prominent I_f pacemaker mechanism. In this subset of cells, the I_f blockers ZD7288 or zatebradine strongly decreased the funny current[174], as monitored under voltage clamp, and led to a bradycardia and a depolarization of the maximum diastolic potential (MDP), which is frequently associated with a cessation of the pacemaker activity[173]. Similar concentrations of zatebradine were previously reported in hESC-CMs[175] or rabbit SAN, and, interestingly, a suppression of the MDP notch could already be observed in those studies but without a clear explanation[176]. The automaticity of those cells was not affected by KB-R7943 or the peptide FRCRCFa, two structurally different inhibitors of the NCX exchanger[177, 178]. In the second subset of cells, although an I_f current could be easily recorded, its full blockade did not affect the spontaneous firing rate as monitored under voltage clamp. By contrast, the hexapeptide FRCRCFa or KB-R7943 strongly decreased the beating rate and depressed the pacemaker activity, resulting in the inhibition of the AP. Interestingly, the depolarizing effect of KB-R7943 on the cell automaticity was previously reported in cardiomyocytes isolated from 8.5 days rodent embryos[167] and in guinea pig SAN cells[179] but the mechanism was not explained. This "I_f-independent" pacemaker mechanism was observed in a subset of cells that represented 41% of the hESC-CMs[173]. In the two cell subpopulations, the "membrane clock" and "calcium clock" were operating in a mutually exclusive manner, although the two conductances coexisted in the same cell and the I_{NCX} current density was higher than that of I_f at MDP[173]. The ubiquitous presence of I_f in all the tested hESC-CMs is also observed in early cardiogenesis[2, 3] and confirms the primi-

tive embryonic phenotype of these cells. The selection of a privileged pacemaker mechanism does not correlate with the relative current densities of I_f and I_{NCX} and remains completely unknown[173]. In the remaining 26% of the hESC-CMs tested, both I_f and I_{NCX} blockers decreased the firing rate by less than 50%. A cumulative negative chronotropic effect on the rate was observed when the cell was subsequently exposed to both types of antagonists [173]. These cells exhibited a "coupled" pacemaker mechanism in which the two inward currents were involved in the diastolic slope. Similar results were described in canine SAN cells[104].

Identification of SK4 channels in hESC-CMs, a previously unrecognized player in the cardiac pacemaker function

Various studies have reported the expression and function of BK and SK channels in heart cells from various animal species. Although BK channels have been thought to be absent from the sarcolemma, a recent study showed a reduction of the heart rate when rat hearts were perfused with paxilline or iberiotoxin[134]. A subsequent study from the same group reported a lengthening of the diastolic depolarization in BK knockout (*Kcnma1*$^{-/-}$) mice[135]. The decreased heart rate was associated with slowed cardiac pacing due to the elongation of the sinus interval. The action potentials recorded from isolated SAN cells were reduced in BK knockout mice compared with WT animals. The heart rate was also slower in the SAN from the control mice treated with paxilline and iberiotoxin[135]. In addition, BK channel immunoreactivity and paxilline-sensitive currents were identified in the SAN from WT mice[135]. We used the same inhibitors to investigate the effect of BK blockade on the pacemaker but did not observe any effects on hESC-CMs[173].

Recently, the three different isoforms of small conductance Ca^{2+}-activated K^+ channels, SK1, SK2 and SK3, were identified in adult mouse and human hearts[144,145,151]. SK2 channels have been reported to operate in the late repolarization phase of the AP of human and rodent atrial cells. The knockout of the SK2 channels leads to a decrease in the firing rate of the AVN cells, whereas the overexpression of the channel is accompanied by shorter APs with increased frequency[147]. SK2 channels are also thought to maintain atrial fibrillation[148]. Apamin, a selective SK2 blocker, delays AP repolarization by elongating the APD_{50} in isolated mouse AVN and atrial cells at concentrations from 50 to 500 pmol/L[146,147]. We did not observe any effect on the rate, the MDP, or the APD_{50} of the young hESC-CMs, even after prolonged exposures to concentrations up to 1 μmol/L, excluding any involvement of this conductance in the pacemaker mechanism at the young stage of hESC-CMs[173]. Interestingly, we could identify a clotrimazole- and TRAM-34–sensitive K^+ current[173]. These two SK4 channel antagonists induced a depolarization of the MDP and a reversible cessation of the automaticity. Notably, the APD_{50} of the cell was not prolonged, suggesting that this conductance was not involved in the repolarization of the AP but rather was involved in the prolongation of the diastolic depolarization period[173]. This current was identified as an I_{KCa} conductance

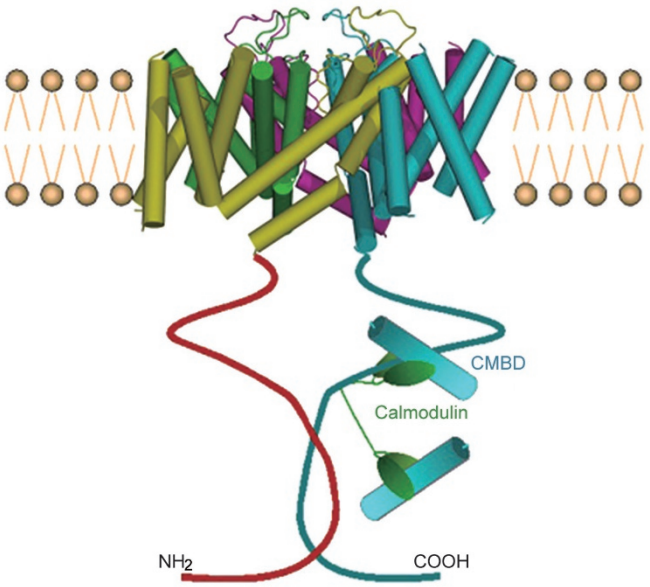

Figure 4. Representative cartoon of an SK4 α-subunit. Each subunit is formed by six transmembrane domains (S1 to S6) with extended cytoplasmic N- and C-termini. S4 is the voltage sensor with few positively charged amino acids. The segment (P-loop) between S5 and S6 forms the pore and includes the "selectivity filter" motif for potassium channels. The N-terminus of one subunit and the C-terminus of an adjacent subunit are shown. The calmodulin binding domain (CMBD) binds Ca^{2+}-Calmodulin, which is necessary for SK4 channel gating.

at the whole-cell current level and also at the single channel level (TRAM 34-sensitive unitary current) with a unitary slope conductance of approximately 21 pS, a value within the range (between 10 to 42 pS) observed for intermediate calcium-activated potassium channels[163]. The molecular identity of the SK4/KCa3.1 channel was confirmed by performing RT-PCR and Western blot analyses on young cardiomyocytes derived from two independent hES lines[173]. Immunocytochemistry for SK4 and α-actinin, a cardiac marker, showed that both proteins were coexpressed in the same cells. Blocking SK4 led to a fast depolarization drift of the MDP and a cessation of the pacing, similar to what was observed when the prominent pacemaker inward current was blocked[173].

Thus, if I_{KCa} is not involved in the repolarization phase of the APs, what is its plausible role in the pacemaker and how does I_{KCa} inhibition account for the MDP depolarization? We hypothesize that as an outward current, I_{KCa} contributes to the MDP driving force by activating the funny current and subtly balancing the inward current of the NCX exchanger during diastolic depolarization. Indeed, I_{KCa} could "shape" the diastolic slope to the intracellular calcium concentration by opposing its outward current to the two depolarizing conductances, I_f and NCX. Whereas the SK1-3 channels have a low affinity for $[Ca^{2+}]_i$ activation (half activation between 300 to 700 nmol/L free Ca^{2+})[136, 137, 139], the SK4 channels exhibit a half activation at 95 nmol/L intracellular free Ca^{2+}, a concentration that is very close to the physiological diastolic $[Ca^{2+}]_i$ level[155].

In guinea pig SAN cells, the minimal diastolic $[Ca^{2+}]_i$ concentration was found to be 225 nmol/L[179], a value that makes the SK4 channels ideally suited for a role at the DD slope period. At the end of the repolarization, the intracellular free calcium concentrations are sufficiently high to activate the SK4 channels (Figure 5).

According to our model, we postulate that the SK4 outward potassium current, which is responsible for the notch of the MDP, provides a driving force that is sufficiently strong to activate I_f at the early phase of the DD slope (Figure 5). The progressive sodium inflow is accompanied by a calcium outflow where NCX operates in its forward mode. The progressive decrease in the intracellular Ca^{2+} concentrations gradually weaken the SK4 K^+ current and allow the I_f and/or I_{NCX} depolarizing inward currents to take over until the threshold for a novel AP is achieved. By contrast, SK4 blockade with drugs such as clotrimazole or TRAM-34 remove the outward current necessary for the DD slope gradient, eventually suppressing the pacing.

How can we explain the depolarizing drift observed after I_f or I_{NCX} blockade? Several studies showed that a blockade of I_f by ZD7288 or ivabradine was associated with a reduction in the intracellular calcium sparks. These observations were made in mouse embryonic (E8–E9) cardiomyocytes[180], in cardiomyocytes isolated from the rabbit pulmonary vein[181], and in rabbit SAN cells[182]. One can assume that the decreased

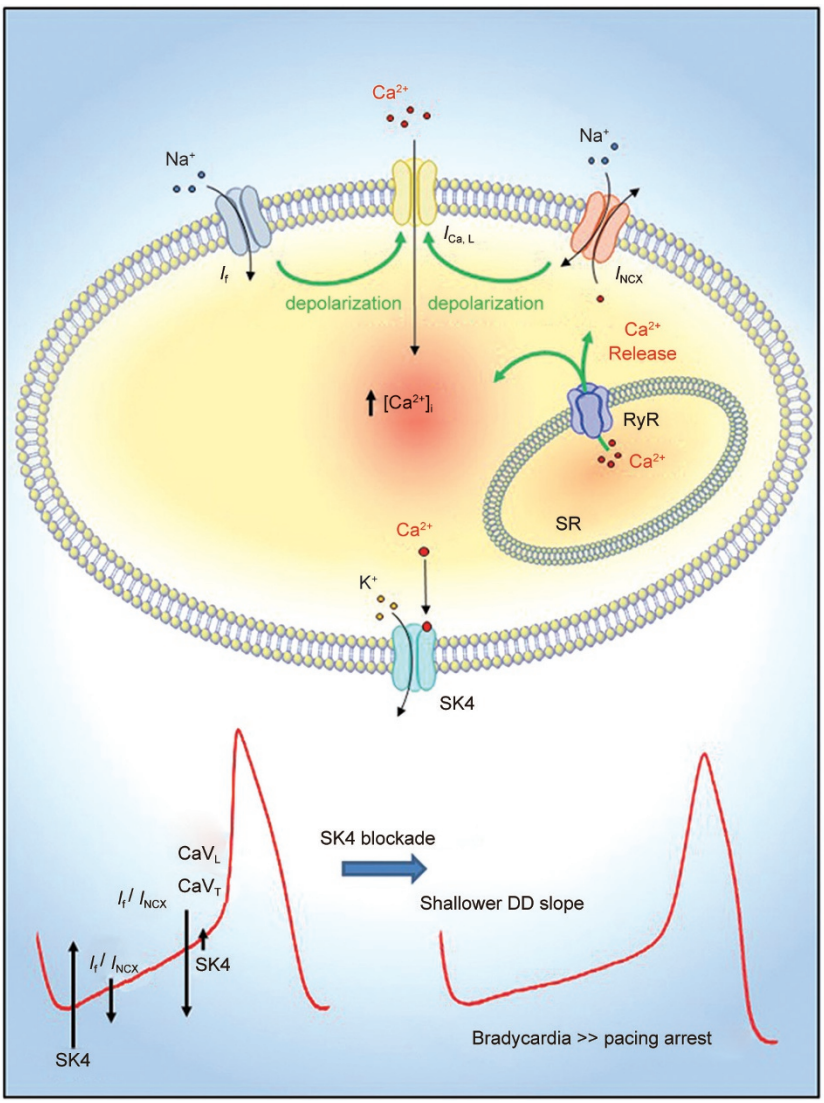

Figure 5. Pacemaker model of human embryonic stem cell-derived cardiomyocytes (adapted from Weisbrod *et al*, 2013, Ref [173]). During the early phase of DD there is enough intracellular free $[Ca^{2+}]_i$ to activate the SK4 channels (I_{KCa}), which contributes to the MDP driving force and concurrently activates I_f. The I_f and NCX currents triggered by the cyclical Ca^{2+} release from the sarcoplasmic reticulum (SR) depolarize the membrane to activate the L-type Ca^{2+} channels ($I_{Ca,L}$) and cause AP cycling. The progressive decrease in $[Ca^{2+}]_i$ from early to late DD mediated by the NCX-induced Ca^{2+} outflow gradually reduces the I_{KCa} currents and allows the inward currents to take over and reach the next AP threshold. Thus, the SK4 channels (I_{KCa}) finely tune the inward currents of the "voltage" and Ca^{2+} clocks and have a crucial role in shaping the DD and the pacemaker activity.

Na^+ influx upon I_f blockade stimulates the forward mode of the NCX exchanger and thereby enhances Ca^{2+} outflow to ultimately reduce the DD cytosolic $[Ca^{2+}]_i$. The decreased $[Ca^{2+}]_i$ eventually inhibits the I_{KCa}, which, together with the enhanced electrogenic activity of NCX, depolarizes the MDP and inhibit the APs. By contrast, I_{NCX} inhibition with equimolar replacement of the external sodium with lithium is accompanied by a reduction in the cytosolic $[Ca^{2+}]_i$[179]. Similarly, we suggest that NCX blockade (*eg*, by KB-R7943 and FRCRCFa) prevents the depolarizing electrogenic activity of NCX and the subsequent activation of the L-type Ca^{2+} channels operating at the late DD period. This action ultimately reduces the DD cytosolic $[Ca^{2+}]_i$, thereby leading to I_{KCa} closure, MDP depolarization, and the suppression of pacemaker activity.

Future direction

SK4 transcripts are expressed in the mouse SAN, adult human right atrium and in ventricular biopsies. Furthermore, the expression of the SK4 protein is restricted to the human right atrium (unpublished data). Similar to the hESC-CMs, we also identified TRAM34-sensitive SK4 currents in the mouse SAN (unpublished data). Overall, our data are consistent with recent studies showing that the SK4 channels are critical players in determining the cardiac pacemaker fate in embryonic stem cells and induced pluripotent stem cells from mice[183–185] and humans[186]. Treatment with 1-ethyl-2-benzimidazolinone (EBIO), the SK4 channel opener, differentiates mouse embryonic stem cells into cardiomyocytes, with a strong enrichment of the pacemaker-like cells[183]. This differentiation is accompanied by the induction of SAN-specific genes and by a loss of the ventricular-specific gene program[183]. Notably, the SK4 channel blocker clotrimazole inhibits EBIO-induced cardiogenesis and the up-regulation of the pacemaker transcripts[183]. In addition, a transcriptional analysis showed a ninefold up-regulation of the SK4 mRNA in the developing conduction system compared with SK1-3[187]. In future studies, it will be crucial to demonstrate the presence of the SK4 channels in mammalian SAN cells, including humans, and their involvement in the maintenance of the cardiac pacemaker. More importantly, the role of SK4 channels should be investigated in idiopathic sinus arrhythmias and also in all cardiac disorders that manifest an impaired sinus rhythm.

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