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MINI-REVIEW

Review: HCN Channels in the Heart

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Abstract: Pacemaker cells are the basis of rhythm in the heart. Cardiovascular diseases, and in particular, arrhythmias are a leading cause of hospital admissions and have been implicated as a cause of sudden death. The prevalence of people with arrhythmias will increase in the next years due to an increase in the ageing population and risk factors. The current therapies are limited, have a lot of side effects, and thus, are not ideal. Pacemaker channels, also called hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, are the molecular correlate of the hyperpolarization-activated current, called I_h (from hyperpolarization) or I_f (from funny), that contribute crucially to the pacemaker activity in cardiac nodal cells and impulse generation and transmission in neurons. HCN channels have emerged as interesting targets for the development of drugs, in particular, to lower the heart rate. Nonetheless, their pharmacology is still rather poorly explored in comparison to many other voltage-gated ion channels or ligand-gated ion channels. Ivabradine is the first and currently the only clinically approved compound that specifically targets HCN channels. The therapeutic indication of ivabradine is the symptomatic treatment of chronic stable angina pectoris in patients with coronary artery disease with a normal sinus rhythm. Several other pharmacological agents have been shown to exert an effect on heart rate, although this effect is not always desired. This review is focused on the pacemaking process taking place in the heart and summarizes the current knowledge on HCN channels.

Keywords: HCN channels, hyperpolarization-activated current, automaticity, sinus node dysfunction, atrial fibrillation, ivabradine.

1. INTRODUCTION

Cardiovascular diseases result in the death of 17.9 million people every year. Based on data from the World Health Organization (WHO), the total annual number of deaths from noncommunicable diseases (NCDs) is projected to increase to 52 million by 2030 [1]. Due to an increase in the ageing population and risk factors, such as tobacco use, unhealthy diet, physical inactivity, and harmful use of alcohol, among others, it is more important than ever to focus on cardiovascular drugs.

Pacemaker channels or hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, designated as HCN1–4, are the molecular correlate of the hyperpolarization-activated current, called I_h (from hyperpolarization) or I_f (from funny), that contribute crucially to the pacemaker activity in cardiac nodal cells and impulse generation and transmission in neurons [2, 3]. The name "funny current" arose due to HCN channels displaying a set of specific biophysical properties that set them apart from most of the other ion channels. For instance, HCN channels activate upon hyperpolarization and deactivate upon depolarization. Moreover, their activation curve shifts to more positive potentials following the binding of cAMP to the channel [4]. HCN channels are permeable for both sodium and potassium ions with a reversal potential near -35 mV [5].

Interestingly, the pharmacology of HCN channels is still rather poorly explored compared to many other voltage-gated ion channels or ligand-gated ion channels. Nonetheless, HCN channels have emerged as interesting targets for the development of drugs, in particular, to modulate the heart rate. The properties of atrial and ventricular I_f, and its modulation by pharmacological interventions, have been the subject of intense study, including the synthesis and characterization of compounds able to preferentially block HCN1, HCN2, or HCN4 isoforms. Altogether, these studies have provided important insights for future pharmacological strategies exploiting the unique properties of this channel family: the prevalence of different HCN subtypes in organs and tissues, the possibility to target HCN gain- or loss-of-function associated with disease, the feasibility of novel isoform-selective drugs, as well as the discovery of HCN-mediated effects for old medicines.

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Ivabradine is the first and currently the only clinically approved compound that specifically targets HCN channels [6]. The therapeutic indication of ivabradine is the symptomatic treatment of chronic stable angina pectoris in patients with coronary artery disease and a normal sinus rhythm [7, 8].

This review focuses on the pacemaking process taking place in the heart. We first briefly summarize the current knowledge of the structure and the biophysical properties of HCN channels. We then discuss the molecular mechanisms underlying the regulation, modulation and function of HCN channels, and their role in human diseases by focusing on heart failure (HF) and arrhythmias. Finally, current pharmacology in chronic heart failure will be discussed.

2. STRUCTURE

Four different genes encode for different isoforms (HCN1–4). Topologically, HCN channels are shown to have an overall structure similar to voltage-gated potassium (Kv) channels. Just as in Kv channels [9-11], four HCN subunits assemble to form a functional channel. The subunits can form different homotetramers or heterotetramers with distinct biophysical properties [12, 13]. Each subunit consists of a cytosolic amino (N)-terminus, a core region, and a cytosolic carboxy (C)-terminus. The most important domain is the channel core, composed of six α-helical transmembrane (TM) domains (S1–S6), with two forming the pore (S5–S6) and four forming the voltage sensor domain (S1–S4) [12].

The pore domain (PD) is formed between segments S5 and S6, and contributes to the central pore. The last transmembrane domain is followed by a cytoplasmic region, which includes a cyclic nucleotide–binding domain (CNBD), where cAMP and cGMP can bind to the channel [14]. This domain is highly conserved, in contrast to the C-terminal part of the C-terminal domain, which shows strong sequence variation among the HCN isoforms. The CNBD is connected to the S6 segment *via* an intracellular C-linker. The binding of cyclic nucleotides to the CNBD induces conformational changes that facilitate channel opening by removing channel inhibition, leading to faster opening kinetics and a shift of the voltage dependence towards more positive values. However, the open probability of HCN can be increased by cAMP, but unlike cyclic nucleotide-gated (CNG) channels, cyclic nucleotides are not a prerequisite for channel opening [5, 12, 15-17]. Moreover, Lee and MacKinnon described another cytoplasmic region, which is unique for HCN. This HCN domain contains 45 amino acids, forming a 3-α-helical domain. It precedes the S1-helix and is wedged between the voltage sensor and the cytoplasmic domains. Since it connects to the S4 helix (near the short S4–S5 linker) from the same subunit and the C-linker and CNBD from an adjacent subunit, it is thought to play a role in the unusual gating behaviour of HCN channels [12].

The S1 to S4 segments form the voltage-sensing domain (VSD), located peripherally to the pore region. The VSDs in HCN1 are ‘‘non-swapped’’, which means that each voltage-sensing domain contacts the pore through amino acids from the same polypeptide chain (*i.e*., the same subunit) [12]. This is in contrast to Kv channels (Kv1–Kv9) [18] and voltage-dependent sodium (Nav) and calcium (Cav) channels, where the voltage sensors interact with an adjacent subunit (*i.e*., they are domain-swapped) [12]. Structural studies using Kv channels have shown that in this canonical model of VSD–PD coupling, the activation energy in the S4 segment of the VSD passes to the S4–S5 linker via the covalent linkage, and subsequently, the S4–S5 linker is formed with the PD *via* a noncovalent interaction between the S4–S5 linker and the S6 to open the pore gate. Since these channels have domain-swapped VSDs, they must have a long α-helical S4–S5 linker [19, 20]. In HCN1, the S4–S5 linker is much shorter and not α-helical [12].

In addition, the VSD comprises an S4-helix with a regular sequence of positively charged amino acids (lysine and arginine), very similar to depolarization-activated channels [21, 22]. The S4 is thought to function as the voltage sensor by moving charge through the membrane electric field in response to depolarization [23]. In comparison to Kv channels, mammalian HCN channels have a much larger S4 domain, containing 9 basic residues and 1 serine residue at every third position [21]. Regardless of having opposite gating mechanisms, both types have a S4 segment moving upward during depolarization and downward during hyperpolarization [23].

Several studies have proposed that the S4–S5 linker plays an essential role in the HCN channel gating mechanism [12, 24, 25]. However, the exact gating mechanism remains debatable. More recent work suggests that the reversed polarity of HCN channels can be caused by the extraordinary length of the S4 helix. Compared to other voltage-dependent ion channels, the S4 helix of HCN1 contains two additional helical turns on the intracellular side. Because of its length, the S4 segment connects to a C-linker in the cytoplasm to stabilize a closed pore when the voltage sensor is depolarized. In addition, the S4, S5 and S6 helices seem to be closely packed together, enabling the depolarized voltage sensor to stabilize a closed pore. Finally, the HCN domain stabilizes the closed pore in the setting of a depolarized voltage sensor. According to this model, the upward movement of the S4-helix has an inhibitory effect on the pore opening. When the membrane potential is hyperpolarized, the voltage sensor moves downward and disrupts these stabilizing interactions, allowing the S6 helices to spontaneously open [12]. Flynn *et al.* mutated the S4–S5 linker, the covalent linkage between the VSD and PD [26], and Cowgill *et al.* systematically developed an array of mosaic channels to display the full voltage-activation phenotypes observed in voltage-gated ion channels [24]. The results showed that hyperpolarization-dependent activation does not require the S4–S5 linker nor the extra length of the HCN S4-helix [24, 26]. In contrast, they did confirm that the gating of HCN channels is inconsistent with the canonical model of VSD–PD coupling in Kv channels [12, 24, 26]. The most recent study by Ramentol *et al.* made an attempt to fully understand the HCN gating mechanism [25]. They identified two residues that appeared to play a crucial role in the polarity of HCN1, namely W355 (part of the QWE motif in S4) and N370 (S5). When W355 and N370 mutate individually, current conditions are detected and the membrane is depolarized. For both the W355N and N370W channel mutants, a 25% conductance remains at positive voltages. This finding suggests that these mutations destabilize the closed pore, and thus, cause

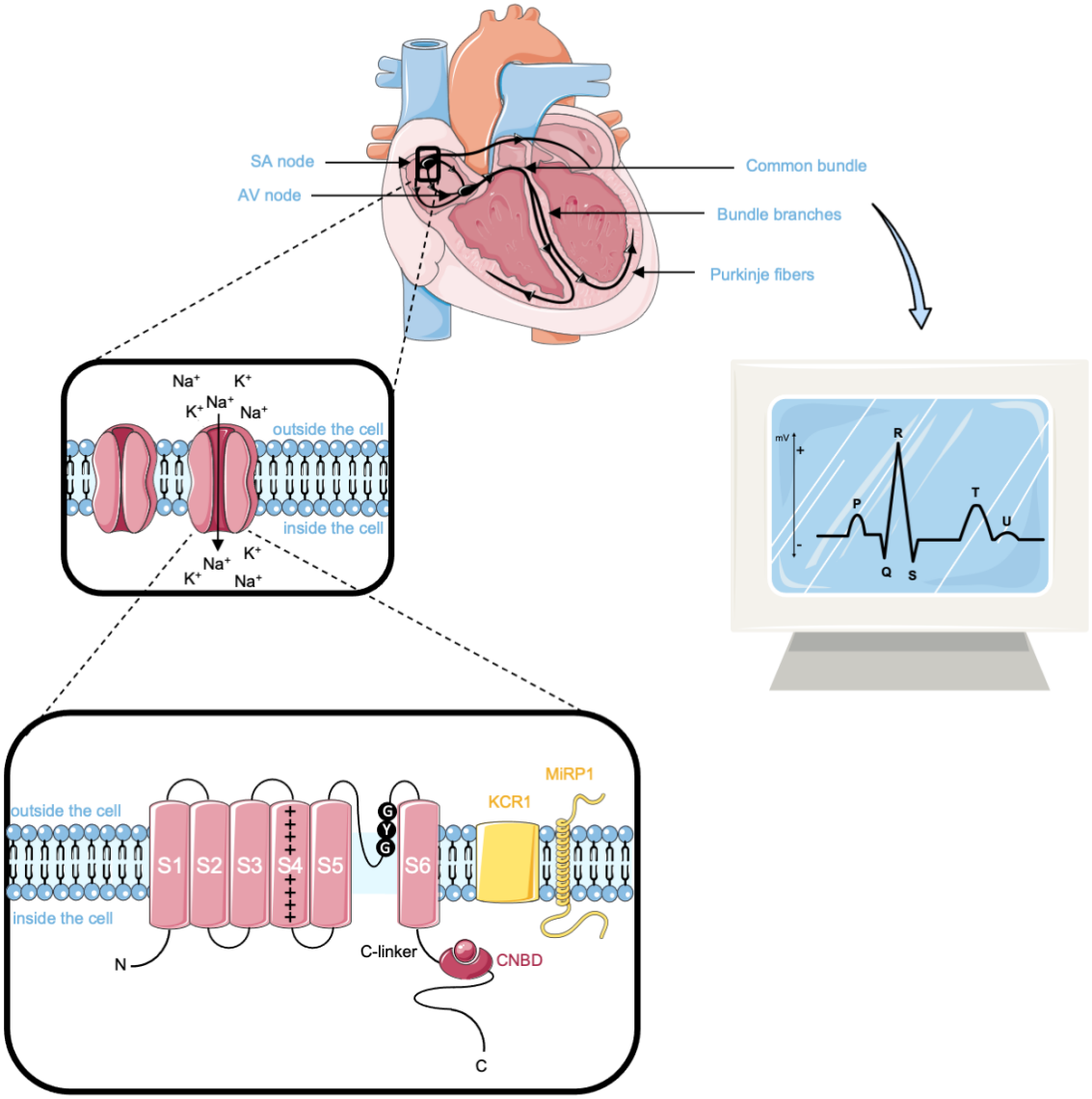

Fig. (1). The location, biophysical properties, transmembrane topology of HCN channels, and regulation of HCN channels by interacting proteins. Figure adapted from Servier Medical Art by Servier. https://smart.servier.com/ (Creative Commons Attribution 3.0 Unported License.) (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

partial opening. Finally, mutation of both the residues (W355N–N370W) could reverse the voltage dependence of HCN channels with no change in the pore structure. This double mutation removes stable interactions, indicating that small differences in the energy of the closed and open states can determine the channel's polarity [25].

Different regulatory proteins form macromolecular complexes with HCN channel subunits and define HCN channel function. For example, the MinK-related peptide 1 protein (MiRP1; encoded by KCNE2) enhances protein and current expression in the cardiac tissue. MiRP1 can cause an upregulation of the channels and alters the kinetics of the various HCN isoforms [27-29]. Mutations in this KCNE2 gene are related to cardiac disorders, including cardiac arrhythmias [30]. Another accessory subunit interacting with HCN channels is Caveolin-3 (CAV3), which is known to affect the correct function of the HCN4 current in the heart [31, 32] (Fig. **1**).

3. BIOPHYSICAL PROPERTIES

HCN channels are members of the voltage-gated pore-loop channel superfamily and are related to the CNG channels and the Kv channels Kv10–Kv12 [5]. Unique among voltage-gated channels, HCN channels are activated when the membrane potential becomes more negative. Upon activation, the inwardly directed cation current slowly depolarizes pacemaker cells to the threshold for action potential (AP) firing. The mixed Na^+ and K^+ permeability of HCN channels is essential for their function as membrane-depolarizing channels, yielding a reversal potential of -20 to -30 mV under physiological conditions [33, 34]. Surprisingly, the selectivity filters of HCN channels are similar to Kv channels since the essential amino acids required for selecting K^+, the common GYG-motif, are conserved among both the channels [12]. Despite the similarity at the molecular level, the HCN channel has a Na^+/K^+ permeability ratio of 1:3 to 1:5 [15-17, 34], whereas Kv has a ratio of 1:1000 [35].

In GYG-containing Kv channels, the selectivity filter sequence is 'T/S-V/I/L/T-GYG', forming four binding sites that allow potassium ions to pass through the ion pore [36, 37]. Since earlier studies showed that (i) large cations, such as Ba^{2+} or tetraethylammonium, do not block I_f, in contrast with Kv channels [17, 38] and (ii) the typical Thr/Ser residue in Kv channels is mutable to Cys (specific to HCN) without affecting the selectivity [36], HCN channels were thought to have a functionally wider pore. A better understanding of the molecular basis of these intriguing biophysical properties was recently obtained by determining the cryogenic electron microscopy (EM) structures of the human HCN1 channel [12]. In this study, the selectivity filter of HCN1 channels was compared to the filter of a distantly related K^+ channel, namely the KcsA potassium channel. It was found that in two of the four filters sites, the Tyr-sidechain in the GYG sequence of HCN1 reorientates to 180 degrees. As a result, the carbonyl oxygen atoms of the peptide backbone that would form two ion binding sites to coordinate K^+ ions are no longer facing the ion pathway in HCN1, leading to a selectivity filter only preserving two binding sites. Since two tyrosine residues bind to one potassium, the HCN pore will only bind one potassium. Single ion binding pores allow more space, which is kinetically favorable for passing sodium [12].

4. REGULATION AND MODULATION

HCN channels are activated by hyperpolarization of the membrane potential; however, this activation can be influenced by cyclic nucleotides interacting with the CNBD, which is located in the intracellular C-terminal region. cAMP accelerates channel opening and shifts the activation curve to a more positive potential [15-17, 39]. The shift in $V_{1/2}$ depends on the channel subtype and the conditions. It varies between 10 and 25 mV for HCN2 and HCN4, but only 2–6 mV for HCN1 [40, 41]. The EC_{50} of cAMP ranges between 0.06 mM and 1.53 mM. Surprisingly, HCN3 is not activated by cAMP; the activation curve is even slightly shifted to more negative voltages [42, 43]. While the HCN channel is inhibited at depolarized potentials, the binding of cAMP removes this inhibition. This induces conformational changes and increases the open probability of the channel pore [40, 44]. Lee and MacKinnon investigated the effect of cAMP by determining the cryo-EM structure of HCN1 in the presence of cAMP. They observed that cAMP binding induces local conformational changes that are propagated from the CNBD *via* the C-linker to the pore, initiating a rotation of the gate-forming inner helices toward the opening. This could explain why cAMP favours HCN channel opening [12]. The reason for the lack of sensitivity of HCN3 to cAMP, despite the presence of a functional CNBD in the intracellular region, is still unknown. Stieber *et al.* suggest the rationale of a shorter C-terminal sequence after the CNBD, which alters the normal autoinhibition of the channel [43].

Another full agonist of HCN channels is guanosine-3',5'-cyclic monophosphate (cGMP). Although cGMP displays a 10-fold lower potency for HCN channels, the shift in $V_{1/2}$ is in the same range as that of cAMP at saturating concentrations [17]. cGMP and cAMP bind to the CNBD in a similar way, with a different orientation of the purine ring [14]. Another modulator of HCN channels is cytidine-3',5'-cyclic monophosphate (cCMP), acting as a partial agonist [39]. On HCN2 and HCN4 channels, cCMP shifts the activation curve to more depolarized potentials and enhances current activation and decreases current deactivation. cCMP does not affect HCN1 and HCN3. The voltage shift and the maximal increase in the current amplitude are significantly smaller than those observed for cAMP, corroborating the behaviour of a partial agonist [45]. Uridine-3',5'-cyclic monophosphate (cUMP), purine-3',5'-cyclic monophosphate (cPMP), and 2-amino-cPMP shift the activation curve to more positive potentials and increase channel conductance for HCN2, with an efficacy similar to cAMP and cGMP. In contrast, inosine-3',5'-cyclic monophosphate (cIMP), as well as cCMP, behave as weak activators [46]. In addition to cyclic mononucleotides, cyclic dinucleotides can also modulate HCN channels. In mouse sinoatrial node (SAN) myocytes, they behave as antagonists, being able to reduce I_h [47]. Cyclic [guanosine-(2'-5')-monophosphate-adenosine-(3'-5')-monophosphate], the cyclic dinucleotide that is found in mammals, caused a shift in the activation curve toward more negative potentials and a 30% reduction of firing rate when 100 μM was added to single SAN cells. For the HCN4 channel expressed in human embryonic kidney (HEK) 293 cells, the dose-response curve yielded an IC_{50} value of 114 nM. Other cyclic dinucleotides could completely reverse the effect of cAMP on the activation curve; for instance, cyclic di-(3',5')-GMP, which is ~16 times less potent than cyclic [guanosine-(2'-5')-monophosphate-adenosine-(3'-5')-monophosphate] (IC_{50} 1.8 mM) [47].

The modulation of HCN channels has been extensively reviewed [48-51]. Besides the cytosolic concentration of cAMP, it is well known that other mechanisms affect channel activity. HCN channel activation also shifts towards depolarizing potentials by phosphoinositides, such as phosphatidylinositol-4,5-bisphosphate (PIP2). It is thought that PIP2 stabilizes the activated state of the voltage sensor [52-54]. In contrast to cAMP, the effect of PIP2 is equal across the four HCN isoforms [53, 55]. Similar to the effect of PIP2, cholesterol has also a regulatory effect on the HCN channels [56, 57]. Cholesterol has been shown to decrease the localization of HCN4 into lipid rafts, leading to a redistribution of the channels within the membrane and modifying the kinetic properties. In addition, it shifted the midpoint of activation to more positive potentials and increased diastolic depolarization in rabbit SAN cells [56]. Fürst *et al.* showed that cholesterol uniquely regulates both channel expression and gating kinetics for each channel isoform [57]. Very recently, Liu *et al.* described a protein, cohesin-protein Shugosin-1 (SGO1), to play a crucial role in maintaining cardiac automaticity [58]. The function of SGO1 is to stabilize the cohesins that are important in the alignment of chromosomes during the G1 stage of mitosis. Next to its influence on cell division, it is shown that SGO1 colocalizes with HCN4. By regulating the HCN4 surface expression, it affects I_f, and thus, pacemaker activity [58]. Furthermore, channels can be activated by an independent mechanism, namely tyrosine phosphorylation by Src kinase. While HCN2 and HCN4 show an accelerated activation upon phosphorylation, phosphorylation does not affect HCN1 [59-62]. Additionally, the HCN channels are activated by bradykinin receptor stimulation, by the activation of phospholipase C, and by hormones released by

the adrenergic and renin-angiotensin-aldosterone system (RAAS) [63]. In the study by Muto *et al.*, they tested the effect of 10 nM aldosterone on cultured neonatal rat ventricular myocytes. The rate of spontaneous pulsing was found to be increased and, in the meantime, an upregulation of the mRNA and protein expression of HCN2 and HCN4 was observed. The latter effect was shown to be abolished by mineralocorticoid receptor (MR) antagonists (eplerenone and spironolactone), reducing the expression levels and currents of HCN2 and HCN4 to a level even lower than in control [64]. These findings were later confirmed by Song *et al.* [65]. Yu *et al.* further investigated the mechanism by which spironolactone regulates HCN channel expression after myocardial infarction (MI). Spironolactone increased miRNA-1 expression in rat ischemic left ventricular myocardium after MI. The upregulation of miRNA-1 (specifically expressed in skeletal muscle and cardiac myocytes) partially contributes to the posttranscriptional suppression of the HCN channel [66].

5. EXPRESSION AND PHYSIOLOGICAL ROLE

HCN channels play a central role in generating cardiac automaticity [67]. In general, HCN transcripts and proteins are expressed at the highest levels in the SAN and the conduction system, including an atrioventricular node (AVN) and Purkinje fibres. In the mammalian heart, the expression levels of individual HCN isoforms vary strongly in different cardiac regions. Since HCN3 shows a weak overall expression, the term "cardiac isoforms" generally refers to HCN1, HCN2, and HCN4 [68, 69]. Also, across different species, the expression levels of individual HCN isoforms differ. Comparable to the SAN of humans, the HCN4 isoform plays a crucial role in automaticity in rabbits, mice, and dogs. Additionally, the HCN1 and HCN2 isoforms can, albeit to a lesser extent, be found in the human heart. On the other hand, only the HCN1 isoform is present in mice and rabbits, and HCN2 can only be found in the SAN of the rat at levels similar to HCN4 [70]. The expression pattern of the HCN protein in the atria and the ventricles also shows isoform variability; however, most mammalians, including humans, exhibit a prevalence of HCN4 and HCN2, followed by a smaller amount of HCN1 and negligible levels of HCN3 [71, 72]. In the human AVN, there is a lack of HCN2 and low expression of HCN1. All three cardiac HCN isoforms have higher expression in the SAN than in the atria. Moreover, since it is almost exclusively present in SAN, HCN1 might be indicated as a selective molecular marker of human SAN and as a potential target of specific treatments intended to modify sinus rhythm [69].

The HCN channels have been recognized for their primary role in the generation and the regulation of pacemaker activity. The specific mechanism of HCN channels has considerably been elucidated in recent years. The role of HCN channels in the SAN is related to the generation and regulation of cardiac pacemaking. However, the relative contribution of the HCN-dependent voltage clock to cardiac automaticity towards the Ca^{2+} clock remains a matter of debate [73-75]. The two main mechanisms, the voltage clock and the Ca^{2+} clock, depend on the HCN current and the Ca^{2+} release from the sarcoplasmic reticulum, respectively. They seem to contribute to a coordinated system that supports spontaneous electrical activity in SAN [76]. In the AVN, the function of HCN channels does not differ substantially. Although the specific role played by HCN channels in this region is less investigated, previous studies have shown that HCN channels are implicated in AVN pacemaking and conduction [77-80]. Atrial and ventricular cardiomyocytes, on the other hand, do not usually display spontaneous activity. These cells have a stable resting membrane potential and infrequently display electrogenesis, consistent with a low contribution of the HCN current to resting membrane potentials (-80/-70 mV) and absence of spontaneous automaticity [63, 81]. A study by Fenske *et al.* showed a different function of HCN in the mouse ventricle. The HCN channel appears to contribute to the ventricular action potential (AP) waveform, specifically during late repolarization. This suggests that HCN channels, and particularly HCN3, provide a background conductance in cardiac myocytes that regulates the resting membrane potential and controls the kinetics of repolarization [82].

Mutations in ion channels and the knockout of ion channels in mice have been linked to sick sinus syndrome or bradycardia. Among these channels, HCN1-deficient mice display congenital SAN dysfunction characterized by bradycardia, and accompanied by low cardiac output, sinus dysrhythmia, and recurrent sinus pauses. This observation confirms the primary role of HCN1 in cardiac pacemaking [83]. Other studies have focused on the physiological role of HCN2. Mice lacking the HCN2 isoform showed pronounced cardiac sinoatrial dysrhythmia. These results reveal that the current generated by HCN2 channels critically determines the resting membrane potential of cardiac pacemaker cells. Nevertheless, the loss of HCN2 does not abolish the spontaneous activity of cardiac pacemaking cells, suggesting that multiple currents underlie pacemaking [84]. Furthermore, transgenic mice overexpressing HCN2 specifically in their hearts (HCN2-Tg) were analyzed. HCN2-Tg mice exhibited no noticeable abnormalities under physiological conditions, except for a significantly faster heart rate. Meanwhile, under pathological conditions, such as an excessive β-adrenergic stimulation, an enhancement in HCN2 channel activity was noticed, reducing the repolarization reserve of the ventricular AP and increasing the ectopic automaticity of ventricular myocytes. These results suggest that, in heart failure, increased HCN2 channel expression alone is not sufficient to induce lethal arrhythmia, but potentially increases the susceptibility to arrhythmias under sympathetic stimulation [85, 86]. Moreover, HCN2 overexpression also increases the vulnerability to arrhythmia under hypokalemic conditions, which is an electrolyte disturbance characterized by an abnormally low level of potassium [87]. Quantitatively, in all vertebrates studied so far, HCN4 underlies the major fraction of SAN current, accounting for 70% to 80% of the total I_f. Mice, deficient in the HCN4 isoform, were used to determine the physiological role of the respective channels. Stieber *et al.* observed mice lacking HCN4 channels, as well as mice with a selective deletion of HCN4 in cardiomyocytes, dying between embryonic days 9.5 and 11.5. While cardiac cells with primitive pacemaker action potentials were found both in wild-type (WT) and HCN4-deficient mice, a mature sinoatrial node-like pacemaker potential did only develop in wild-type embryos starting at day 9.0. This ob-

servation indicates that HCN4 is essential for the formation of mature pacemaker cells during embryogenesis [88]. Another study, conducted by the same research group, showed that deletion of HCN4 in adult mice reduced the sinoatrial I_f, on average by about 75%, and resulted in a cardiac arrhythmia characterized by recurrent sinus pauses. Surprisingly, they noticed that the channel does not play a role in the β-adrenergic-induced increase in heart rate, suggesting that HCN4 functions as a depolarization reserve supporting pacemaking in certain critical physiological states [89]. Transgenic mouse models in the study by Baruscotti *et al.* showed that the selective reduction of the HCN4 current is consistently accompanied by progressive development of severe bradycardia and AV block, eventually resulting in cardiac arrest and death of the animal. These contradicting results may be related to the inducible and cardiac-specific HCN4 knockout mouse model used [90]. Abolition of HCN4 sensitivity through the conditional expression of dominant-negative HCN4 channels lacking cAMP sensitivity reduces the spontaneous activity of AVN cells under basal conditions but does not impair the maximal response to β-adrenergic stimulation. This suggests that HCN4 channels influence basal excitability of mouse AV node cells, but are not required for acceleration of pacemaker activity under β-adrenergic receptor stimulation [78]. On the other hand, overexpression of the HCN4 isoform resulted in mice developing a dilated cardiomyopathy phenotype with increased cellular arrhythmogenicity, while the heart rate and conduction parameters remained unchanged. They did notice a change in the Na^+/Ca^{2+} exchanger equilibrium. Due to the increased I_f, a diastolic Na^+ influx causes an increased intracellular calcium concentration, which in turn creates significantly higher systolic intracellular calcium transients and stimulates apoptosis [91]. Therefore, HCN2 and HCN4 channels can be said to play a crucial role in the normal rhythmicity of sinoatrial myocytes.

6. PATHOLOGY

Atrial fibrillation (AF) is the most common persistent arrhythmia. A well-known risk factor is older age, with approximately 70% of AF patients being 65-85 years old [92]. Li *et al.* investigated the relationship between ageing and changes in HCN channel distribution in the sinoatrial node and atrium. In the SAN of the aged dogs, the expression level of HCN2 and HCN4 channel mRNAs and proteins was lower, while there was an upregulation in the atrium. Aged dogs presented a higher induction rate of AF in response to electrical stimulation, longer AF duration after induction, and longer sinus node recovery time compared to adults [93]. The age-associated expression of the HCN channel isoforms in rat SAN was also investigated by Huang *et al.* The protein levels of HCN2 and HCN4 decreased in 30-month-old rats compared to 4-month-old adult rats, corroborating the age-dependent lower intrinsic heart rate. Additionally, the effect of ivabradine, an HCN channel blocker, on rat SAN automaticity decreased [70]. HCN4 protein expression in aged rats is reduced, not only in the SAN, but also in the bundle of His within the AVN. These results indicate that HCN4 plays an important role in AVN dysfunction with ageing [94].

Another factor associated with an increased risk of arrhythmias is diabetes mellitus (DM). Huang *et al.* used streptozotocin (STZ)-induced diabetic rats to investigate the expression of the four HCN isoforms. The SAN of these animals showed a reduction in transcripts and proteins of HCN2 and HCN4 compared to the age-matched controls. The STZ-induced diabetic rats were characterized by cardiac alterations (lower intrinsic heart rate, lengthened sinoatrial conduction time, and rate-corrected maximal sinoatrial node recovery time *in vivo* as well as a longer cycle length *in vitro*) and by an impaired SAN function (inferior leading pacemaker site, reduced SAN conduction velocity and diastolic depolarization slope, and longer AP duration) [95].

In accordance with the physiological role of the HCN channels, genetic mutations modifying I_f functions are related to the genetic basis of arrhythmias. In humans, these channel mutations are exclusively limited to the HCN4 isoform and rarely to KCNE2 (Fig. **2**). A review by Verkerk and Wilders reported 22 mutations or variants in HCN4 associated with sinus node dysfunction (SND) [96]. However, after evaluation of a clear genotype-phenotype association, this number reduced to 13 [97]. SND, also termed sick sinus syndrome (SSS), is defined as the "intrinsic inadequacy of the SAN to perform its pacemaking function due to a disorder of automaticity and/or inability to transmit its impulse to the rest of the atrium". The heart rhythm can be too fast, too slow, interrupted by long pauses (>2 or 3 seconds), or an alternating combination of these problems [96]. Difrancesco *et al.* stated that all mutations are heterozygous and all mutations are dominant-negative, with various degrees of penetrance. In addition, they mentioned that all are loss-of-function (LOF) mutations. LOF is defined as a functional loss caused either by a negative shift of the activation curve and/or by the lower density of membrane expression of channels, and/or by a consequent reduction in current density and/or cAMP sensitivity variations [96, 97]. Möller *et al.* described two more homomeric channel mutants (R550H and E1193Q) showing LOF through increased rates of deactivation. A third channel mutation, R378C, exhibited a shift to the left in the activation curve and a slower activation rate. Possibly for the three mutants, this was caused by a significantly reduced functional HCN4 channel availability and cell surface expression due to defective trafficking [98]. The E1193Q mutant is located in the distal C-terminus and was previously described in a patient with paroxysmal AF [99]. Genetic screening of patients with suspected or diagnosed Brugada or SSS identified a novel mutation, V492F, located in S6, a highly conserved site of HCN4. Unlike other HCN4 mutants, this LOF can be fully attributed to a reduction in channel activity, while other mutations impact the synthesis and/or trafficking of the channel protein. Heteromeric channels, composed of WT and mutant monomers, show a shift in activation to more negative voltages, partially rescuing the I_f [100]. Among the SND, there is an inappropriate sinus tachycardia (IST). A familial form of IST is associated with a first gain-of-function (GOF) mutation (R524Q), contradicting the initial general observations by Difrancesco *et al. Ex vivo* analysis showed enhanced sensitivity to cAMP, leading to an increased pacemaker current during diastole [101, 102]. In addition to its known association with SND, HCN4 channel dysfunction may play a distinct role in the develop-

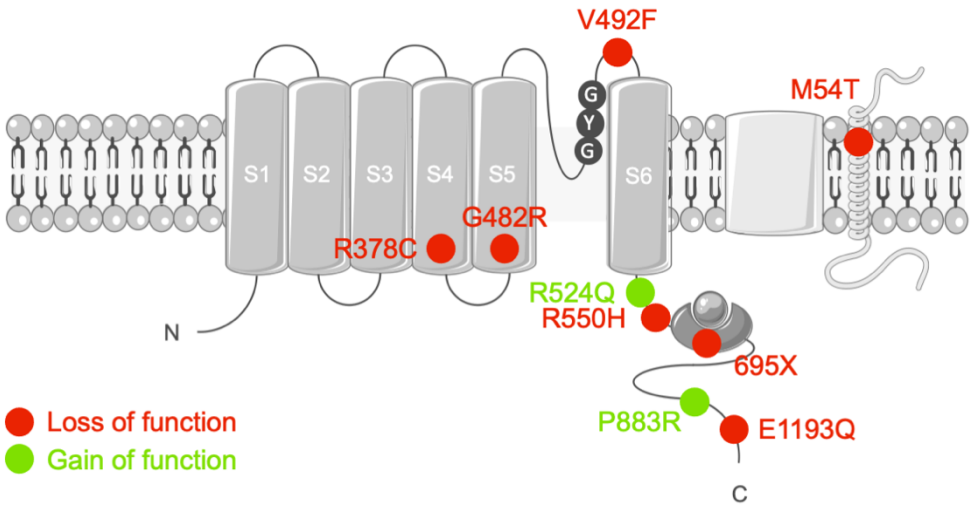

Fig. (2). Mutations in KCNE2 and the HCN4 isoform associated with sinus node dysfunction. Figure adapted from Servier Medical Art by Servier. https://smart.servier.com/(Creative Commons Attribution 3.0 Unported License.) (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

ment of structural cardiac abnormalities. In family members with SND and noncompaction cardiomyopathy (NCCM), a new HCN4 LOF mutation G482R, located in the highly conserved channel pore domain, was identified and segregated with a combined disease phenotype. NCCM is characterized by excessive ventricular hypertrabeculation and complicated by heart failure, arrhythmia, and thromboembolic events. Moreover, due to a different truncation (695X) and a missense (P883R) HCN4 mutation segregated with a similar combined phenotype in an additional, unrelated family and a single unrelated proband, respectively, it can be concluded that HCN4 mutations are also critically involved in the development of NCCM [103]. Weigl *et al.* identified the previously uncharacterized HCN4 mutant, P883R, in three unrelated patients with AF and tachycardia-induced cardiomyopathy (TIC). The P883R mutation, however, was shown to be a new GOF mutation. Unlike the R524Q GOF mutation, P883R channels were characterized by a positive shift of the half-maximal activation voltage, while channel deactivation was faster. Moreover, net sensitivity towards cAMP was reduced. Co-transfection of WT and mutant channel, resembling the heterozygous cellular conditions of the patients, showed significantly higher current densities compared to WT. Altogether, these results could explain the increase in ectopic trigger and maintenance of AF [104].

As indicated earlier, the KCNE2 gene encoding the MiRP1 protein can carry mutations. Nawathe *et al.* identified a 55-year-old patient with the M54T MiRP1 mutation, presenting sinus bradycardia, and further investigated this mutation in human HCN2 and HCN4 isoforms. The voltage dependence of neither HCN2 nor HCN4 was altered by M54T. Additionally, while the current amplitude of HCN4 decreased, no effects were seen on the current amplitude of HCN2. Finally, M54T slows activation kinetics of HCN2 and HCN4 at physiologically relevant voltages but does not alter HCN4 deactivation kinetics [105]. There is evidence to support key roles of HCN-KCNE2 complexes in establishing susceptibility to SND. The expression patterns of KCNE2 in cardiac tissue resemble that of HCN channels, and gene variants alter the stoichiometry and increase the susceptibility to several of the same cardiac disorders linked to HCN, including cardiac arrhythmias [30].

7. PHARMACOLOGY

7.1. Current Controversies Concerning Ivabradine

Ivabradine, developed by Servier®, is the one molecule currently on the market acting as a specific HCN channel blocker. The European Medicines Agency (EMA) approved ivabradine for the treatment of stable angina and the management of patients with HF in 2005 and 2012, respectively. The Food and Drug Administration (FDA) only approved the drug to reduce rehospitalization rates in patients with chronic and stable HF in 2015 [8]. Ivabradine is licensed under different names like Procoralan®, Corlentor®, and Corlanor®, and is prescribed for patients with HF, with an ejection fraction $\leq 35\%$ and a heart rate ≥ 70 bpm. In comparison to other treatments to control the heart rate (HR), such as β-blockers and calcium channel inhibitors, the effect of ivabradine is not associated with common side effects, like hypotension and a negative inotropic effect.

Bucchi *et al.* showed that HCN4 is inhibited by ivabradine in a use-dependent manner, with IC_{50} in the micromolar range. While ivabradine is an open-channel blocker of HCN4, it is a closed-channel blocker of HCN1 channels [106, 107]. Ivabradine exhibits a high selectivity for HCN channels, but various studies have shown that higher concentrations of this drug may also affect other cardiac ion channels [108]. A study by Delpon *et al.* showed a block of Kv1.5 by ivabradine, with an IC_{50} value of 29.0 ± 1.9 μM [109]. Also, Kv11.1 and Nav1.5 are inhibited by ivabradine at micromolar concentrations in heterologous systems [110]. T-type calcium currents are not affected by ivabradine (10 μM), while L-type calcium currents are decreased by <20% after the application of 3 μM of ivabradine. The use of ivabradine is restricted due to the lack of detailed information concerning subtype selectivity among HCN1–HCN4 channels, but it is thought to be a preferential HCN4 blocker [8]. Lack of specific selectivity may explain the side effects,

such as bradycardia, hypertension, atrial fibrillation, and temporary visual disturbances, known as phosphenes [111].

7.2. HCN Channel as a Drug Target

7.2.1. Peptide Toxins

To date, only one peptide toxin is found to modulate HCN channels. Gamma-conotoxin-PnVIIA is described from the venom of the molluscivorous snail *Conus pennaceus* to belong to the six cysteines four-loop structural family of conotoxins [112]. Fainzilber *et al.* purified the toxin and characterized it by conducting electrophysiology experiments. First, caudodorsal neurons from the snail *Lymnaea stagnalis* were used to investigate the effects on action potential firing and membrane potential. It was shown that PnVIIA caused an increased excitability of these neuroendocrine cells and an increased number of action potentials, both in a dose-dependent manner. Besides the dose, the experiments showed that the duration of PnVIIA application influenced the response. The membrane potential was investigated by injecting hyperpolarizing current pulses into the cells, causing hyperpolarization of the membrane potential. Upon application of PnVIIA, the hyperpolarizing response decreased, indicating a decrease in the membrane resistance. They concluded that PnVIIA leads to the opening of ion channels. In the next series of experiments, they used the whole-cell voltage-clamp configuration to investigate the nature of these ion channels. Since no effect was seen on Nav or Cav channels, they believed PnVIIA modulates the pacemaker channels. After the addition of 10 μM PnVIIA, the activation curve shifted to more negative potentials by ~10 mV, and the non-inactivating outward current (at potentials above 0 mV) increased. However, further studies are required to elucidate the mechanism of action [112].

In a case study by Agarwal *et al.*, they reported sinus node dysfunction following a snake bite for the first time [113]. Two hours after the snake bite, a 35-year-old man was admitted to the hospital. As seen in similar cases, the man presented with coagulopathy, an increased prothrombin time, and an increased activated partial thromboplastin time (aPTT). Nevertheless, he was hemodynamically stable, and after a complete check-up, his other parameters seemed normal. His electrocardiogram (ECG), on the other hand, displayed sinus arrest with junctional escape rhythm and retrograde P waves, at a rate of 40 beats/min. After 20 vials of anti-snake venom and 3 days of hospital admission, the ECG showed normal sinus rhythm. They ruled out the diagnosis of toxic myocarditis and suggested that the snake venom caused a modification in the electrophysiological properties of the cardiac cell membrane, and hence, affected impulse generation and conduction. This venom could be explored as a possible source of novel peptide toxins modulating the HCN channel. However, they mentioned that future research is also required [113].

7.2.2. Small Molecules

To date, several pharmacological agents have been shown to exert an effect on the heart rate. An extensive and thorough review of these drugs has been published by Romanelli *et al.* [108]. They summarized the current knowledge regarding the compounds that have been shown to modulate HCN channels, including α2-adrenergic and opioid receptor agonists (dexmedetomidine, clonidine, loperamide, tramadol), general anaesthetics (propofol, ketamine), local anaesthetics, antiarrhythmic drugs (lidocaine, mepivacaine, bupivacaine, dronedarone), and several others [108, 114-116].

There is a great need for novel potent and selective HCN channel inhibitors as the use of ivabradine is limited. Compounds selective for HCN1 have been described by McClure *et al.* One of the compounds is 15 times more selective for HCN1 than for HCN4 [117]. Carvedilol is a third-generation β1-blocker, clinically effective in the treatment of chronic HF and exhibiting favourable effects compared to other β-blockers. HCN4 current significantly reduces when carvedilol is used in the low micromolar range. In addition, these effects are also shown for HCN1 and HCN2, but with a higher IC_{50}. The rate of channel activation is found to decrease, that of deactivation to increase, and the voltage-dependence of activation to shift to more hyperpolarizing potentials [118]. Chen *et al.* developed and synthesized a series of alkanol amine derivatives [119]. The compound 4e blocked the HCN2 current with an IC_{50} value of 2.9 ± 1.2 μM. Its application caused a slowing of activation and a shift to more negative potentials in the voltage dependence of HCN2 channel activation. Attempts at achieving a selective inhibition of individual HCN isoforms have been performed through the design of a series of phenylalkylamines related to zatebradine, which is a known blocker of I_f in cardiac cells [120]. The compounds EC18 and MEL57A showed preferential block for HCN4 and HCN1, respectively. They tested selectivity on HCN channels expressed in HEK cells. Subsequent tests showed that the effect was preserved in native tissues, where the subunits are thought to form heteromeric channels. In guinea pig SAN cells expressing HCN4 channels, only EC18 displayed an effect, while in DRG neurons, where HCN1 is the most relevant contributor to native I_h, only MEL57A exhibited a significant effect. In dog Purkinje fibres, where HCN4 is the prevalent isoform, only EC18 reduced the amplitude and decreased the slope of the diastolic depolarization phase [121-123]. However, since it is thought that HCN1 and HCN2 channels can co-assemble and form heterotetramers, MEL55A shows an interesting pharmacological profile, exhibiting a preferential block for HCN1 and HCN2 over HCN4 [122, 124]. β-blockers have been shown to reduce morbidity and mortality in patients with HF, but the effect on the SAN function is still unclear. Du *et al.* presented a study where bisoprolol improved SAN function in rats with HF by reversing the down-regulation of the HCN4 channel [125]. Also, the mechanism of ethanol on the SAN is still not completely understood. Romanelli *et al.* previously mentioned that the application of ethanol shifts the voltage dependence of HCN channels to more depolarized potentials and increases the maximum conductance, causing an increase in I_f, and thus, in spontaneous firing [108, 126]. These results were confirmed by Chen *et al.* [127]. Recently, the first high-throughput screening of 16000 small-molecule compounds using an automated patch-clamp system was conducted to identify novel HCN4 blockers [128]. Three novel hit compounds were identified, T-478, T-788 and T-524, with IC_{50} values of 4.2 μM, 0.97 μM and 10.3 μM, respectively. All three compounds exhibited different blockade curves. While T-478 showed similar curves to ivabradine, the curves of T-788 and T-524 were

similar to ZD7288. Finally, two compounds, already used for several years as herbal medicine, have been studied on HCN channels. A recent paper by Chan *et al.* suggested honokiol (HNK) as a potential modulator of the HCN channel [129]. HNK is a small-molecule polyphenol obtained from *Magnolia officinalis*, and has been used in traditional Asian medicines. They investigated the effect of HNK on the density and gating of I_h in pituitary tumor (GH3) cells and in Rolf B1.T olfactory neurons. The density of I_h was found to be decreased and the activation curve shifted to more hyperpolarized potentials. The IC_{50} value was estimated to be 2.1 μM. Further experiments are required to investigate the effect of HNK on I_f in cardiac myocytes, where it could possibly reduce the rhythmic activity of the heart. Next, the effect of Ginkgo Biloba Extract (GBE) had been tested on HCN channels [130]. It has been shown previously that GBE decreased the slope of the diastole and inhibited I_f in SAN cells [131, 132]. HCN2 and HCN4 channel currents are inhibited by GBE in a concentration-dependent manner. HCN4 was shown to be more sensitive than HCN2, with IC_{50} values of 0.12 ± 0.05 mg/ml and 0.25 ± 0.01 mg/ml, respectively. This antiarrhythmic mechanism of GBE might be useful for the treatment of arrhythmia.

CONCLUSION AND FUTURE PERSPECTIVE

The evidence that HCN channels are important drug targets in the therapy of several cardiovascular diseases is overwhelming. Dysfunction of HCN channels plays a crucial role in cardiac arrhythmias, ischemic heart disease, and ventricular hypertrophy [133]. Due to their unique features, HCN channels are present in pacemaker cells only, and are emerging as ideal targets for the modulation of pacemaking. The current therapies are rather limited. For years, research has been seeking compounds for lowering the heart rate without the side effects of β-blockers and calcium antagonists. Hence, these HCN channels are seen as a valuable alternative for the development of new clinical therapies. Although ivabradine is clinically approved as a treatment for stable angina pectoris and is a great tool to investigate the mechanism of the HCN channels, it is bound by a major limitation, *i.e.*, it is equipotent across the different HCN channel isoforms. Since non-selective modulation of HCN channels can cause unwanted side effects, future discoveries of novel and HCN channel isoform-selective compounds are required. The currently available substances, including ivabradine, inhibit HCN channels through the same mechanism; they block the channel pore [106, 134]. Since the pore region is extremely conserved across the channel isoforms, the chances of discovering isoform-selective molecules may be higher when targeting other regions of the channel. The recently published cryo-electron microscopy (EM) structure of HCN1, by Lee and MacKinnon, can lay out a framework for structure-guided drug discovery [12]. HCN channel isoform-selective drug design will be facilitated when the cryo-EM structures of HCN2 and HCN4 will be discovered, and subsequently, the differences in the channel structures of the different isoforms could be determined. Furthermore, the cryo-EM structure of HCN1 will provide the means for understanding the mechanism of action of HCN channels.

Future research is required to discover isoform-selective molecules modulating HCN channels, and to further determine the physiological role of HCN channels. This knowledge will be fundamental for the treatment of a variety of HCN channelopathies.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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