HCN Channels and Cardiac Pacemaking

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Abstract

Cardiomyocytes located in the central part of the sinoatrial node are responsible for generating the electrical rhythm of the heart since they are endowed with the fastest automaticity of the entire conduction system. The source of this automaticity is the diastolic pacemaker phase which consists of the slow depolarization that links the end of each action potential with the beginning of the next, and the funny current ("I_f") is the primary contributor of this phase. Each f-channel results from the assembly of four single subunits belonging to the family of the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels which includes four isoforms (HCN1–HCN4). The biophysical and modulatory properties of the f/HCN current will be presented together with some of the underlying molecular details which have been partly unraveled by the recent structural definition of the channel obtained by cryo-electron microscopy studies. The chapter will also provide an extensive review of the mutations of the HCN4 channels in humans associated with sinus arrhythmias and left ventricular noncompaction cardiomyopathy. Functional studies based on HCN transgenic and knockout mouse models confirm the importance of the I_f current in sustaining the pacemaker activity since its suppression affects the cardiac performance and autonomic modulation of heart rate. These studies also provide the evidence that cardiac HCN currents are required for proper cardiac development and embryo survival.

Finally, the clinical relevance of HCN channels as targets of drugs aimed to selectively reduce the heart rate will be also discussed.

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5.1 Spontaneous Activity of Sinoatrial Node Cells and the Native "Pacemaker"

The first medical observation that the heart has an intrinsic automaticity which persists also when the heart is removed from the body was made by Claudius Galenus in the second century AD, but only in 1907 the structure responsible for the initiation of the heartbeat, the sinoatrial node (SAN), was identified by Keith and Flack (Silverman et al. 2006; Keith and Flack 1907).

SAN cells are specialized myocytes which generate rhythmic action potentials (APs) that spread along preferential routes (Conduction System tissue) to the entire heart to trigger the orderly sequence of contractions of the heart chambers. Sinoatrial APs differ from those of the atrial and ventricular working myocytes in many aspects, but the most relevant is the lack of a stable resting potential since after SAN cells have reached the maximum diastolic potential (MDP, around −60 mV), the membrane slowly depolarizes up to the threshold (around −40 mV) for the initiation of a new AP; in so doing this phase sets the time interval between consecutive APs and thus the heart rate. This slow depolarization is commonly known as "pacemaker" phase, phase 4, or slow diastolic depolarization (DD, Fig. 5.1a, left). Several of the molecular details of the fascinating puzzle of electrical events that generate the pacemaker activity of SAN cells and allow our heart to beat and sustain our metabolic needs have now been identified (Mangoni and Nargeot 2008); in this chapter we will focus on the role of the pacemaker "I_f" current (Fig. 5.1a, right). The I_f current, discovered in 1979 in rabbit SAN cells (Brown et al. 1979), has the following features: (1) activation in hyperpolarization, (2) mixed Na^+/K^+ permeability, and (3) modulation by the second messenger cAMP; these aspects will be here briefly discussed (for a more accurate treatment, see DiFrancesco et al. 1986; Baruscotti et al. 2005).

1. *Activation in hyperpolarization.* The I_f current has the unusual property of being activated on membrane hyperpolarization rather than on depolarization, and the activation and deactivation kinetics are S-shaped with an initial "delay" followed by the real "gating" process (DiFrancesco and Ferroni 1983; DiFrancesco 1984). The reported values of its voltage dependence are largely variable with activation threshold and half-activation (V½) values in the range of −35/−70 mV and −52/−90 mV, respectively (Baruscotti et al. 2005). Such a large scattering of the data, which is unusual for most other ion channels, may be accounted for by several causes, both biological and methodological. A real biological heterogeneity is indeed caused by the intrinsic differences in the voltage dependence of the I_f current recorded in different areas of the SA node since it activates at progressively more negative voltages when moving from the center to the periphery (Boyett et al. 2000). In addition, the presence of "run-down," a well-known phenomenon which progressively reduces the I_f current and shifts its availability curve during patch-clamp recordings, should also be considered (DiFrancesco et al. 1986). Although a comprehensive molecular understanding of the run-down process is still incomplete, part of it is due to the depletion of f-channel

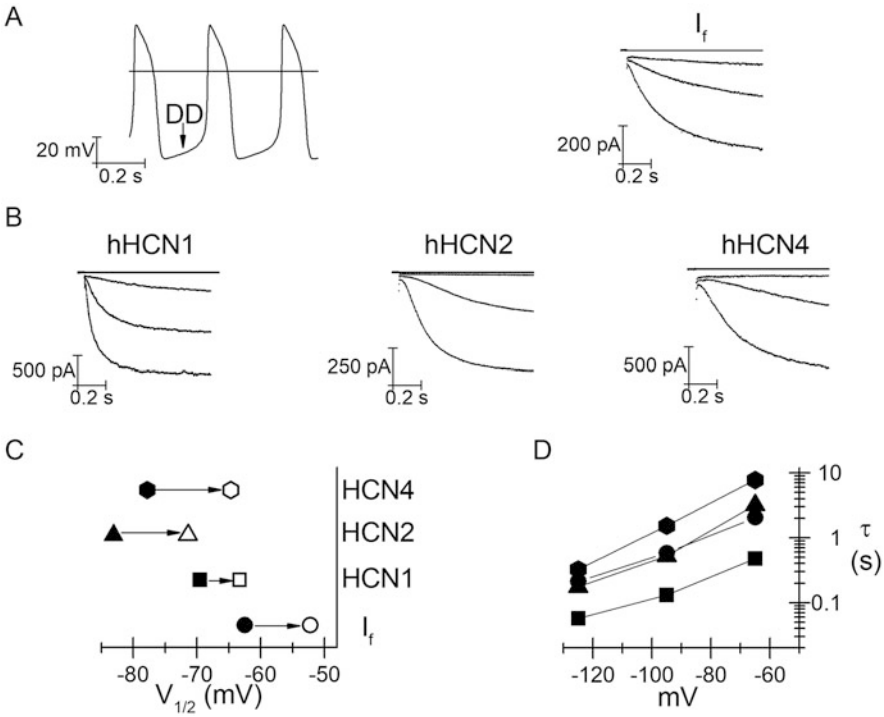

Fig. 5.1 Properties of native sinoatrial pacemaker (f) and homotetrameric HCN currents. (**a**) Sample action potentials recordings (left) and native I_f current traces (right) recorded in rabbit SAN cells. Current traces were acquired during voltage steps at −65 mV, −95 mV, and −125 mV (hp −35 mV). (**b**) Sample hHCN1, hHCN2, and hHCN4 clonal current traces elicited by voltage steps at −65 mV, −95 mV, and −125 mV (hp −35 mV). (**c**) Comparison of half-maximal activation values (V½) of native I_f, hHCN1, hHCN2, and hHCN4 currents in control conditions (filled symbols) and in the presence of saturating concentrations of cAMP (open symbols). Both V½ values obtained in control condition and the cAMP-induced shifts display significant differences (data obtained from Altomare et al. 2003; Stieber et al. 2005; Moroni et al. 2000; Baruscotti et al. 2017). (**d**) Comparison of activation time constants of HCN currents and native I_f. Activation time constants were obtained by fitting current traces in A with a single exponential function after an initial delay

modulators, such as cAMP and phosphoinositide PI(4,5)P2, which occurs during whole-cell patch-clamp recordings (Pian et al. 2006). Finally, the different experimental conditions and recording protocols used by different laboratories may also represent an additional source of variability of the observed differences in the voltage dependence.

2. *Mixed Na^+/K^+ permeability.* f-channels are permeable to both Na^+ and K^+ ions with a reversal potential around −10/−20 mV (DiFrancesco and Ojeda 1980; DiFrancesco 1981b; DiFrancesco et al. 1986); thanks to the recently obtained 3D resolution of the channel structure, the structural molecular elements governing

this mixed selectivity have now been identified (Lee and MacKinnon 2017). This Na^+/K^+ permeability is fundamental for the generation of the diastolic depolarization phase, indeed; although the channel is ~3.7 to fourfold more permeable to K^+ than to Na^+ ions (DiFrancesco 1981b; Frace et al. 1992), the inward and depolarizing Na^+ flux prevails at diastolic voltages (about −40 to −60 mV in the SAN).

3. *Modulation by the second messenger cAMP.* The control of f-channels kinetics by the second messenger cAMP represents an important physiological mechanism used by the neurohormonal system to adapt the cardiac chronotropism to the metabolic demand of the body (Brown et al. 1979; DiFrancesco and Tromba 1987, 1988; DiFrancesco et al. 1989). In SAN cells the stimulation of β-adrenoreceptors (β-ARs) by catecholamines activates the stimulatory G protein (Gαs) and the adenylyl cyclase (AC) leading to the increase in cAMP cell content. cAMP molecules are direct modulators since they can bind to the f-channels and in so doing exert a modulatory action which favors the equilibrium toward the open state which can be quantitatively described as a shift of the activation curve toward more positive voltages (Fig. 5.1c) (DiFrancesco and Tortora 1991; DiFrancesco and Mangoni 1994). This molecular event ultimately results in an increased inward f-current and a steeper diastolic depolarization and therefore a cardiac acceleration (Bucchi et al. 2007). According to Barbuti et al. (2007), SAN cells express both β1- and β2-AR subtypes; however, β2 stimulation determines a more relevant shift of the I_f current and consequently a more pronounced rate acceleration than β1 stimulation. These functional results are nicely paralleled by the evidence that membrane microdomains such as caveolae are rich in β2-ARs and f-channels, while β1 receptors are mainly outside these membrane regions (Barbuti et al. 2007). SAN cells are also abundantly innervated by vagal terminals which release Acetylcholine (ACh). In the presence of a cholinergic stimulus, the muscarinic receptors activate the inhibitory G protein (Gαi) which then inhibits the AC, therefore, ultimately leading to the following set of events: a decrease in cAMP levels, a shift of the activation curve of f-channels toward more negative potentials, and a decline of both the pacemaker current and heart rate.

   The I_f current is not only expressed in SAN cells, but it is also present in other cardiac regions such as atrioventricular node (AVN) cells and Purkinje fibers, where it activates at more negative voltages compared to SAN myocytes (Munk et al. 1996; Hancox et al. 1993; DiFrancesco 1981a, b). The presence of both spontaneous activity and of the I_f current has also been reported in cells isolated from the region surrounding the rabbit tricuspid valve and from canine and rabbit pulmonary sleeves that are extensions of the left atrial myocardium into the pulmonary veins (Anumonwo et al. 1990; Chen et al. 2000, 2009; Suenari et al. 2012). While the functional roles of these currents are still unexplored, it is interesting that AV blocks were observed in association with knockout of the cardiac HCN4 channel in a mouse model (Baruscotti et al. 2011); also interesting is that the pulmonary veins are often the target of the ablation procedure in human patients suffering from atrial fibrillation, raising the question whether anomalous

HCN-dependent activity may represent a molecular and functional derangement contributing to AF.

Taken together all these data strengthen the association between the presence of spontaneous activity and the expression of the pacemaker current.

5.2 Clonal HCN Currents

Clonal and native f-channels have a tetrameric composition, with single subunits belonging to the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family, and in mammalians four isoforms (HCN1–HCN4) have been identified (Ludwig et al. 1998; Santoro et al. 1998; Baruscotti et al. 2010). Each HCN subunit has intracellular N- and C-termini and a central core domain organized in six transmembrane segments (S1–S6), with a positively charged S4 acting as the voltage sensor, and a pore region between S5 and S6 carrying the GYG signature typical of K^+-permeable channels (Fig. 5.2). A cyclic nucleotide-binding domain (CNBD) is localized in the C-terminus and is connected to the S6 segment by the C-linker (see next section for structural details). The primary structures of the four HCN isoforms are 80–90% identical in the transmembrane core and in the CNBD but diverge in the amino- and carboxy-terminal cytoplasmic regions (Viscomi et al. 2001).

1. *Biophysical and modulatory properties:* Heterologous expression and in vivo studies have shown that the different HCN isoforms can assemble both as homotetramers and heterotetramers (with the exception of HCN2-HCN3 heteromers, Much et al. 2003) to yield functional channels with properties similar to native f-channels: mixed Na^+ and K^+ permeability, activation upon hyperpolarization, time-dependent activation and deactivation, and modulation by the second messenger cAMP. Despite these qualitative similarities, heterologous expression of homotetrameric channels has revealed that different isoforms exhibit important quantitative differences in their kinetic aspects and in cAMP modulation. For example, HCN1 channels exhibit the more positive position of the activation curve followed by HCN4, HCN3, and HCN2 (Baruscotti et al. 2010), while the activation kinetics become progressively slower according to the following order: HCN1, HCN2, HCN3, and HCN4 (Fig. 5.1b–d).

   cAMP-induced modulation of f/HCN currents represents a key process by which the autonomic nervous system fine-tunes the slope of the diastolic depolarization of SAN cells and therefore SAN activity and heart rate (DiFrancesco 1993). As it will be discussed more thoroughly in the next section of this chapter, cAMP binding to CNBD stabilizes the open state of the channel gate, and, in kinetic terms, this action results in a positive shift of the activation curve which is larger for HCN2 and HCN4 than for HCN1 (Sartiani et al. 2017; Baruscotti et al. 2005; Chen et al. 2001) (Fig. 5.1c). According to a large part of the literature, the opening of HCN1 channels is only weakly facilitated by the binding of cAMP molecules. However, this interpretation has recently been challenged by some authors who suggest that HCN1 channels are stably associated with cAMP

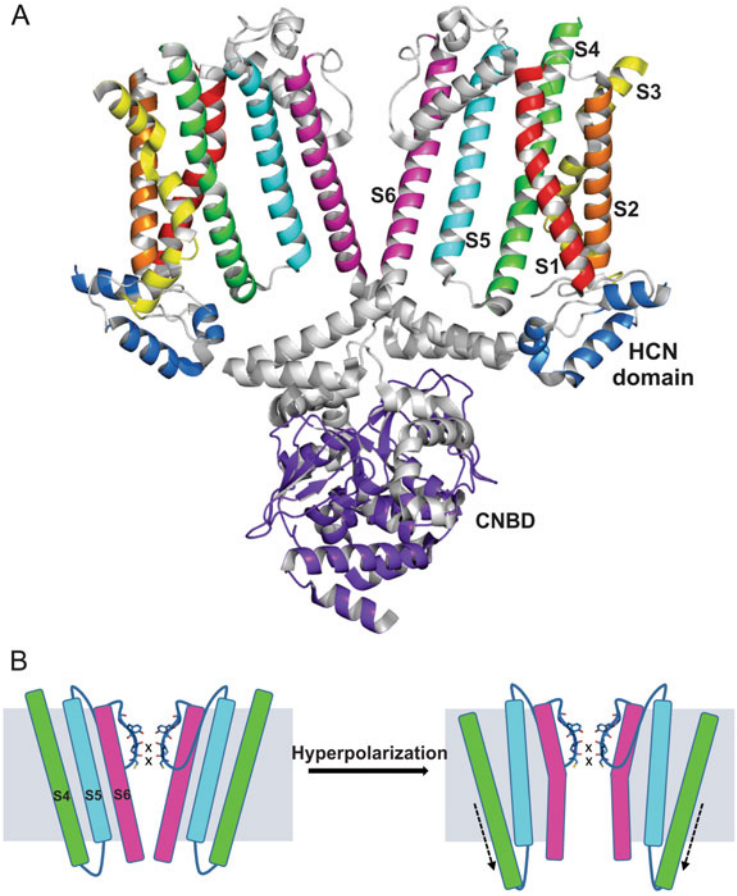

Fig. 5.2 Structural organization of the HCN1 channel. (**a**) Structure of the HCN1 channel (pdb 5u6p) obtained by Lee and MacKinnon (2017). Only two of the four subunits are shown for clarity. The transmembrane segments (S1, S2, S3, S4, S5, and S6), the cyclic nucleotide-binding domains (CNBD), and the HCN domains are indicated. (**b**) Schematic representations of the S4-S5-P-S6 regions of the HCN1 channel in the closed (left) and open form (right), derived from the cryo-EM structures of hHCN1 (Lee and MacKinnon 2017). Only two of the four subunits are shown for clarity. Residues lining the selectivity filter are shown in stick mode, and the only two cation binding sites are represented as x. Upon hyperpolarization, the downward displacement of the S4 helix drives a series of conformational changes in the subunits allowing the S6 helices to open (see text for details)

molecules (Lolicato et al. 2011; Chow et al. 2012); according to this view, any further increase in cAMP concentration would not induce any additional modulation thus resulting in an apparent weak modulation. Interestingly, this hypothesis could also account for the evidence that the activation curve of HCN1 channels is the most positive among those of HCN isoforms. Finally, cAMP stimulation of HCN3 is peculiar and unclear since it has been described as a lack

of or a small negative shift (−2.9/−5 mV) (Stieber et al. 2005; Mistrik et al. 2005).

Heterologous expression of various isoforms revealed that heteromeric channels possess kinetic and modulatory properties intermediate between those of the individual components (Ishii et al. 2001; Chen et al. 2001; Ulens and Tytgat 2001; Altomare et al. 2003; Much et al. 2003). Although at present a detailed understanding of the structural and functional mechanisms responsible for differences in the voltage dependence and in the time course of activation is still missing, there is evidence indicating that the C-terminus region contributes to these processes. Indeed, replacement of the HCN4 C-terminus by that of HCN1 caused a strong acceleration of activation and deactivation rates and a decreased response to cAMP (Viscomi et al. 2001).

2. *Tissue distribution in the adult heart and during development.* Molecular investigations have reported the presence of various HCN isoforms in mammalian cardiac conduction tissue; however, their distribution and relative expression are extremely different and differently developmentally regulated. In the human adult healthy heart, HCN1, HCN2, and HCN4 proteins are highly expressed in the SAN, and mRNA transcripts of *Hcn1*, *Hcn3*, and *Hcn4* have been detected in Purkinje fibers (PF) (Gaborit et al. 2007; Chandler et al. 2009; Li et al. 2015). Several studies have identified the presence of HCN4 channels (both transcripts and proteins) in the atrioventricular node (AVN) and pulmonary veins of different species including humans, thus providing a molecular identification for the f-currents that are normally recorded in these cells at physiological voltages (Greener et al. 2009, 2011; Dobrzynski et al. 2003; Li et al. 2014; Ye et al. 2015; Yamamoto et al. 2006).

Although HCN1 and HCN4 are the main isoforms in the mammalian SAN (Chandler et al. 2009; Li et al. 2015; Brioschi et al. 2009), their expression either alone or in combination failed to reproduce the sinoatrial I_f current. Several molecular mechanisms may account for this discrepancy, and they are collectively referred to as "context dependence" (Qu et al. 2002); the term context dependence intends to highlight the fact that the ultimate functional behavior of the channel also depends on the interacting and accessory proteins (i.e., Mink-related protein (MiRP) 1, caveolin3, KCR1, and SAP97), phosphorylation state, membrane phospholipid (i.e., phosphatidylinositol 4,5-bisphosphate, PIP_2), surrounding membrane composition and fluidity, interaction with cyclic dinucleotides (c-di-GMP and 2′3′-cGAMP), and likely other yet unidentified mechanisms (Baruscotti et al. 2010). Small amounts of HCN2 and HCN4 proteins have also been detected in the adult human atria and ventricles, while HCN1 were noted only in the atria (Chandler et al. 2009; Stillitano et al. 2008; Li et al. 2015).

Despite the fact that pacemaker currents are functionally irrelevant in healthy atria and ventricles because of their small densities and negative, nonphysiological threshold for activation (Porciatti et al. 1997; Hoppe and Beuckelmann 1998; Hoppe et al. 1998), we are now aware that kinetic alterations and/or overexpression of these channels is often observed in association with

cardiac disease such as chronic AF, heart failure (Stillitano et al. 2008, 2013; Cerbai et al. 2001; Hoppe et al. 1998). These pathology-related alterations of HCN expression are likely associated with the arrhythmic profiles often observed in these conditions.

Several studies have indeed elegantly shown that, in addition to generating the pacemaker activity in the adult heart, f-currents are also critical for a correct cardiac development. Indeed in the mouse embryo, significant levels of *Hcn4* mRNA can be detected as early as embryonic day (ED) 7.5 in the cardiac crescent (Garcia-Frigola et al. 2003). As development progresses (ED8), *Hcn4* can be found in ventricular progenitors of the first heart field which drive the peristaltic contraction of the heart tube (Liang et al. 2013), even though these cells will not form the mature sinus node. From ED9.5 the expression of the HCN4 channels will be progressively restricted to the sinus venous, the region that will become the adult SAN. Only few data on the expression of other HCN isoforms in the developing heart are available: Stieber et al. (2003), for example, showed that in global HCN4 knockout mice at ED9.5, the HCN1 and HCN3 isoforms are expressed and may account for the residual I_f current recorded in embryonic cardiomyocytes.

5.3 Structural Hallmarks of HCN Channels

HCN channels have been the focus of intense molecular investigation aiming at the identification of the structural domains associated with specific functional features as mentioned previously. The recent resolution of the cryo-electron microscopy structure of the human HCN1 in the closed state has substantially advanced our knowledge in this field (Lee and MacKinnon 2017). The 3D structure has indeed provided a structural interpretation of the following aspects: (1) the mixed permeability of HCN channels, (2) the activation upon hyperpolarization, and (3) the cAMP-induced facilitation of the close-to-open transition. We will now briefly review the main aspects of this structure-function association.

1. *Mixed Na^+/K^+ permeability of HCN channels.* The ionic selectivity of tetrameric HCN channels is determined by the presence within the hairpin-shaped pore region comprised between the S5 and S6 transmembrane segments of four GYG triplets which form the selectivity filter (SF) of K^+ channels. A puzzling question that has long remained unanswered was why the GYG arrangement of K^+ channels restricts the permeability to K^+ ions ($P_{Na}/P_k > 1000:1$), while HCN channels are instead highly permeable to Na^+ ions [P_K/P_{Na} of 2–5:1 (Ludwig et al. 1999; Santoro et al. 1998; Moroni et al. 2000)]. The cryo-EM structure has elegantly shown that the outer half of the SF of HCN channels is more dilated than that of purely K^+ selective channels, and this forces the SF filter of HCN channels to have only two cation binding sites instead of the four present in pure selective K^+ channels

(Fig. 5.2b). The presence of four binding sites in K^+ channels allows the simultaneous coordination in the SF of two ions with the consequence that since K^+ ions are more stably coordinated than Na^+ ions, the presence in the SF of a K^+ ion opposes the permeation of Na^+ ions limiting the permeability to K^+ ions. On the other hand, the structural organization of the SF of HCN channels limits to one the number of ions that the SF can "host" at the same time, thus allowing a mixed Na^+/K^+ permeability (Lee and MacKinnon 2017).

2. *Activation upon hyperpolarization.* Pacemaker channels are hyperpolarization-activated channels, and this rather unique property is caused by an unusually long S4 segment which, at depolarized potentials, protrudes in the cytoplasm (Fig. 5.2b). The consequence of the anomalous S4 segments is that at depolarized potentials the S4–S5 linkers interact and stabilize the four C-linkers (C-linker disk) in a position which forces the activation gate at the bottom of the S6 helices (HCN1: Val390, Thr 394, and Gln 398) to assume a tight (closed) conformation. During membrane hyperpolarization, the S4 segments are driven further in the cytoplasmic direction, and this displacement ultimately twists and releases the constraint of the S6 segments that are now free to assume a more energetically stable conformation corresponding to the open state of the channel. A novel information provided by cryo-EM investigation is the presence of a particular domain in the N-terminus which is unique to HCN channels and for this reason is called "HCN domain" (Fig. 5.2a). This domain, composed by the 45 amino acids preceding the transmembrane segment S1, and arranged in three α-helices, takes contact with both the S4 helix of the same subunit and the C-linker of the adjacent subunit and acts as an additional element that determines the structural stability of the closed channel.
3. *cAMP-induced facilitation of the close-to-open transition.* Binding of cAMP molecules to the CNBDs induces a structural rearrangement of these domains and the rotation of the C-linker elements; this movement determines a small displacement of the S6 segments and the opening of the channels (Lee and MacKinnon 2017). In other words, cAMP binding contributes to remove channel inhibition. It is interesting to note that functional experiments have previously shown that membrane hyperpolarization and cAMP binding are allosteric partners in regulating the opening processes (DiFrancesco 1999; Altomare et al. 2001) and it is thus likely that the structural movement and final state of the gate induced by voltage are identical to those occurring upon cAMP binding.

5.4 Pathophysiological Aspects of HCN Channel Mutations

The results obtained with in vitro studies on the role of pacemaker channels provided the logical background for genetic studies aiming at the identification of HCN channel mutations associated with inheritable cardiac impulse generation and conduction dysfunctions. This search has been extremely fruitful for the *HCN4* isoform, while no pathological mutations in *HCN1*, *HCN2*, or *HCN3* genes have been so far reported. In the following part, we will discuss the HCN4 disease-causing mutations

according to their position within the different structural and functional domains of the channel.

5.4.1 Mutations of the N-Terminus (P257S)

In a recent study, Macri et al. (2014) searched for the presence of HCN4 mutations in patients with early-onset atrial fibrillation and identified seven novel variants (seven in the AF population and three in the control population with no history of AF): p.K189R, p.P257S, p.T822M, p.G885R, p.P945S, p.G1077S, and p.E1193Q. Expression studies of these mutations were carried out in CHO cells, and, with the exception of the P257S located in the N-terminus, no functional differences in current densities and kinetic features were found. When homomerically expressed, the P257S variant did not yield any measurable currents likely because mutant channels were retained in the cytoplasm as revealed by immunocytochemistry. Thus, it was concluded that the P257S mutation disrupts membrane trafficking. Whether in native cardiac cells this trafficking defective mechanism leads to a reduced current expression is a possibility that could explain the clinical features observed in the single patient carrying the heterozygous mutation.

5.4.2 Mutations of the S4 Segment (R393H) and of the S4–S5 Linker (A414G)

5.4.2.1 R393H

The mutation p.R393H (c.1178G>A) has recently been identified by Ishikawa and coworkers in three family members presenting different types of cardiac dysfunctions: bradyarrhythmia (proband), sinus arrest and supraventricular escape beats (brother), and dilated cardiomyopathy and atrial fibrillation (father) (Ishikawa et al. 2017). Heterologous in vitro expression of mutant channels in tsA-201 cells resulted in a marked reduction of the current (∼−77% for homomeric R393H/R393H and ∼−57% for heteromeric wt/R393H), but trafficking defects were excluded. Half-activation values (V½) and time constants of activation did not differ between heteromeric and wt currents, while a significant difference was found in the slope factors. The authors also report that cAMP-induced modulation was maintained in heteromeric channels, but it could not be evaluated in homomeric channels.

5.4.2.2 A414G

Milano et al. identified the mutation p.A414G (c.1241C>G), located in the S4–S5 linker, in three related individuals (father and sons) variously affected by sinus bradycardia and left ventricular noncompaction cardiomyopathy (LVNC), AF, and atrial standstill (Milano et al. 2014). Patch-clamp studies in transiently transfected CHO cells showed that the voltage dependence of heteromeric wt/A414G channels was negatively shifted by 23.9 mV, thus causing a significant reduction of the

current density in the pacemaker range of potentials (−50/−60 mV). These data, together with the established notion that the S4–S5 linker is an important structural element that couples voltage-dependent movement of the S4 segment to the gating structures, support the conclusion of a causative association between the p.A414G mutation and the bradyarrhythmic phenotype observed in mutant carriers. The presence of LVNC has also been reported in association with the p.695X and p.883R HCN4 mutations (Schweizer et al. 2014; see also below). Although a clear understanding of this association is still missing, Milano et al. hypothesize that LVNC may be congenital or secondary to sinus bradycardia (Milano et al. 2014). During embryonic heart development, HCN4 channels are expressed in progenitors of the first heart field (that will later contribute to the formation of the working myocardium; Barbuti and Robinson 2015); for this reason dysfunctional embryonic HCN4 currents may be the direct cause of the structural alterations observed in the adult cardiac tissue. Alternatively, LVNC may represent a remodeling process which is caused by the chronic bradycardia.

5.4.3 Mutations of the HCN4 Selectivity Filter (G480R, Y481H, G482R)

A structural and functional hallmark of HCN channels, as well as of all members of the extended K^+ superfamily, is the presence in the selectivity filter of the conserved GYG motif (in HCN4 G480-Y481-G482) whose role has been discussed in one of the previous sections. Genetic investigations have identified inheritable HCN4 channelopathies associated with the following loss-of-function mutations: p. G480R (Nof et al. 2007), p.Y481H (Milano et al. 2014), and p.G482R (Milano et al. 2014; Schweizer et al. 2014; Ishikawa et al. 2017).

Clinical data show that patients carrying either the mutation p.Y481H or p. G482R are commonly affected by sinus bradycardia and LVNC (Milano et al. 2014; Schweizer et al. 2014; Ishikawa et al. 2017). Other phenotypes such as mitral valve prolapse, syncope, AF, and first-degree AV block have also been variously reported. Heterologous expression of Y481H and G482R mutant channels allowed to define these mutations as loss of function, because they both determine a large decrease of the current density (Milano et al. 2014; Schweizer et al. 2014). Different results are reported for the effects of the mutations on the voltage dependence: according to Milano et al. (2014), the mutations p.Y481H and p.G482R induce large negative shifts of the activation curves (V½: wt/wt −68.4 mV, wt/Y481H −112.3 mV, wt/G482R −107.1 mV), while no significant shift was found by Schweizer et al. (2014) (V½: wt/wt −94.6 mV, wt/G482R −91.5 mV). Since the V½ values of wt HCN4 currents reported by the two groups differ by about 26 mV, any attempt to interpret the differences in the voltage dependence of mutant channels is difficult.

The clinical phenotypes associated with the p.G480R mutation are much less disruptive [asymptomatic sinus bradycardia and no structural heart abnormalities (Nof et al. 2007)]. Expression studies in HEK cells showed that homomeric G480R

currents were practically absent (~90% reduction), while heterologous expression of heteromeric wt/G480R channels yielded two separate set of cells: one with currents identical to the wt and the other with currents similar to the homomeric G480R expression. Western blot experiments carried out in the heterologous expression system confirmed an extremely faint signal for homomeric p.G480R mutant proteins. It is certainly puzzling that while expression studies indicate a striking phenotypic effect, individuals carrying the p.G480R mutation only display a mild bradycardia. Of particular interest is the observation that among all HCN4 channel mutations, only those affecting the pore residues G481 and Y482 are also associated with LVNC. Although at present only speculative, this may reflect a defective role of HCN4 channels during embryonic development (leading to structural alteration of ventricular tissue) rather than a consequence of the altered pacemaker activity. As illustrated in the previous section, the structure of the selectivity filter of the HCN1 channel has been identified by means of the cryo-electron microscopy (Lee and MacKinnon 2017); under this simplified assumption, the residues G480 and Y481 should form the bottleneck of the selectivity filter, while residue G482 is more externally rotated and should not contribute to ionic coordination (Fig. 5.2; see also the previous section). Under this simplified assumption, the mutation of the residue G482 should have a smaller functional impact on the phenotypic clinical aspects than mutations of the residues G480 and Y481. This obviously is not the case, and the reasons for this dichotomy are unclear.

5.4.4 Mutations of the P Region-S6 Extracellular Loop (A485V, V492F)

5.4.4.1 A485V

Laish-Farkash identified the presence of the p.A485V (c.1454C>T) mutation in three individuals affected by symptomatic sinus bradycardia and normal structural heart conditions (Laish-Farkash et al. 2010). The first patient was hospitalized for cardiac arrest during intense exercise, the second patient for pre-syncopal events, and the third one for paroxysmal atrial fibrillation. Although the individuals were unrelated, they all shared a Moroccan-Jewish heritage, thus suggesting a common origin of the mutation. Bradycardia, dizziness, and pre-syncopal events were largely present in other family members carrying the mutation. Current densities elicited by homomeric and heteromeric A485V expression in oocytes and HEK293 cells were severely reduced, and Western blot experiments in A485V-transfected HEK 203 cells confirmed that mutant proteins were ~50% less than wild-type channels.

5.4.4.2 V492F

The heterozygous mutation p.V492F causing a substitution of the hydrophobic valine with the aromatic phenylalanine has recently been identified in a patient with suspected Brugada syndrome by Biel et al. (2016). While the hHCN4 V492 is highly conserved for the HCN4 isoform, this residue is absent in HCN1 and HCN2 isoforms where it is substituted by leucine (hHCN1) and isoleucine (hHCN2)

hydrophobic residues. Expression of mutant heteromeric channels in HEK cells reduced currents which retained cAMP modulation, while homomeric expression resulted in virtually no currents. Whether this mutation is responsible of the Brugada syndrome and why no sinus arrhythmias were observed is an open question.

5.4.5 Mutations of the C-Linker (R524Q, K530N, D553N, 573X)

The C-linker region, which in the hHCN4 channel extends from residue S522 to residue A599, connects the S6 transmembrane domain to the cyclic nucleotide-binding domain. The C-linker is not only a structural bridge, but it is also a functional key domain since in the presence of cAMP, the "C-linker disk," formed by the assembly of the four C-linkers, rotates and in doing so favors the opening of the channel gate. Several mutations have been identified in this region.

5.4.5.1 R524Q

We recently identified the first HCN4 gain-of-function mutation (p.R524Q) in siblings of an Italian family affected by the inappropriate sinus tachycardia (IST, Baruscotti et al. 2017); IST is a rare clinical syndrome, and diseased individuals exhibit a faster than expected cardiac rate both at rest and during moderate physical activity (Baruscotti et al. 2016, 2017; Codvelle 1939; Sheldon et al. 2015; Vedantham and Scheinman 2017). The residue p.R524 is a highly evolutionary conserved arginine which is positioned in the A′ α-helix of the C-linker region of HCN channels. In the homomeric wild-type channel, the four p.R524 residues assemble as a positively charged ring located in a region where multiple interactions between the S4–S5 linker, the S5 helix, and the C-linker are at work to control channel gating. Heterologously expressed homomeric R524Q channels have a ~21-fold higher sensitivity to the second messenger cAMP (dose-response Kd values of 0.08 and 1.67 μM for R524Q and wild-type channels, respectively) but no difference in their intrinsic voltage dependence (measured in inside-out condition in the absence of cAMP). Since cAMP and membrane hyperpolarization exert an allosteric control of pacemaker channel availability, an increase in cAMP sensitivity ultimately results in an increased I_f current flowing during diastole, therefore leading to tachycardia (Fig. 5.3a) (Baruscotti et al. 2017).

Our study did not address the structural reason for the increase in cAMP sensitivity, however it provided additional support to the conclusion that the C-linkers are structural elements central to the modulation of the channel.

The concept of cAMP affinity for HCN channels is not only central to the interpretation of the role of the R524Q mutation, but it could also represent a more general molecular mechanism of IST. Indeed, there is evidence that a large fraction (~50%) of IST patients have β-adrenergic receptor autoantibodies, a condition that causes a larger-than-normal cAMP production (Chiale et al. 2006) and, hence, pacemaker channel activation. Taken together these studies support the concept that nonphysiological cAMP overproduction and increased HCN sensitivity to cAMP are molecular mechanisms responsible for IST.

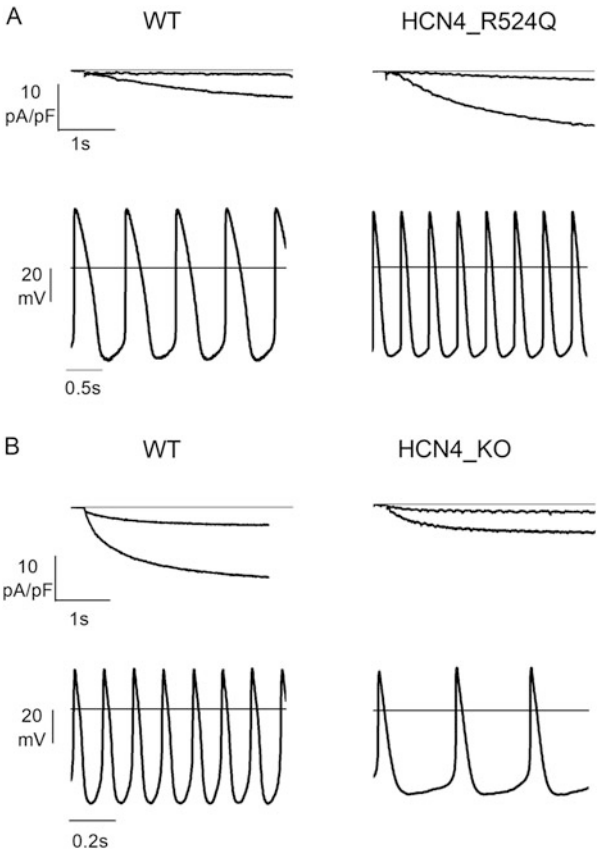

Fig. 5.3 The I_f current density influences the spontaneous rates of rabbit adult SAN myocytes and rat newborn cardiac myocytes. (**a**) The gain-of-function HCN4 R524Q mutation increases both the pacemaker current and the spontaneous activity of rat newborn cardiac myocytes. Sample current traces (steps to −55 and −75 mV, hp = −35 mV; top), and APs (bottom) recorded from rat newborn cardiac myocytes overexpressing either the WT (left) or the R524Q mutated (right) channels. (**b**) The knockout of HCN4 channels decreases both the I_f current and the spontaneous activity of SAN cells. Sample I_f traces (steps to −55 and −75, hp = −35 mV; top) and APs (bottom) recorded from SAN cells isolated from control (left) and HCN4 KO (right)

5.4.5.2 K530N

The heterogeneity of the phenotypic manifestations of mutations of the C-linker region is further demonstrated by the clinical signs associated with the K530N mutation which was identified in members of a family affected by mild asymptomatic sinus bradycardia, age-dependent tachy-brady syndrome, and persistent atrial fibrillation (Duhme et al. 2013).

Similarly to p.R524Q, the p.K530N mutation is located in the A′ helix of the C-linker and replaces a positively charged lysine with an asparagine. Expression studies of mutant channels unexpectedly showed that the K530N mutation had a significant impact on the functional properties of the channel only in the heteromeric condition. For example, V½ of activation were wt, −87.5 mV; K530N/K530N, −88.8 mV; and wt/K530, −101.7 mV, while cAMP-induced V½ shifts were wt, +14.3 mV; K530N/K530N, +17.4 mV; and wt/K530, +21.8 mV. Given the heterozygous condition of the affected carriers, we could speculate that while the negative shift of the voltage dependence could account for the bradycardic condition, the increased response to cAMP stimulation may represent the mechanisms associated with the tachycardic aspect of the tachy-brady syndrome.

Since K530 residues are involved in the subunit-subunit interaction, the authors proposed that in the heteromeric condition, residues N530 likely alter the integrity of this interaction and cause an inhibitory action on the voltage-dependent gating (i.e., favor the closed state). However, when all four K530 residues are mutated (homomeric mutants), proper channel gating is maintained.

5.4.5.3 D553N

The heterozygous mutation p.D553N was originally discovered by Ueda and collaborators (Ueda et al. 2004) in three members of a family affected by severe bradycardia and QT prolongation; recurrent syncope and polymorphic ventricular tachycardia were also observed in the proband. The electrophysiological properties of mutant channels have been analyzed by heterologous expression in COS7 cells: a large reduction of the current density (~−65%−75% and ~−92% for heteromeric and homomeric channels, respectively) and no alterations of the voltage dependence were reported (Ueda et al. 2004; Netter et al. 2012). However, while Ueda et al. (2004) observed a trafficking impairment with cytoplasmic retention of the channels, this was not reported by Netter et al. (2012). Although a thorough investigation was not carried out, Netter et al. (2012) showed that homomeric D553N channels failed to be modulated by high cAMP concentration.

5.4.5.4 573X

The first mutation ever identified in the *HCN4* gene was the single-base 1631delC deletion found in a single index patient. This mutation, located in the C-linker coding region, caused a frameshift and an early stop codon resulting in the truncated protein 573X lacking part of the C-linker, the CNBD, and the C-terminus (Schulze-Bahr et al. 2003). The clinical characterization included the following symptoms: sinus bradycardia, episodes of syncope, intermittent atrial fibrillation, and chronotropic incompetence. Lack of cAMP-dependent modulation was reported both in the homomeric and in the heteromeric channels suggesting a dominant negative action, while immunofluorescence experiments indicated normal trafficking to the plasma membrane. The 573X mutation represents a milestone for HCN4-related channelopathies, and although a conclusive causative association between the genotype and the clinical phenotype of this mutation was impossible because it was based on a single patient, we can now retrospectively state that this association has been clearly supported by all the findings that followed its discovery.

5.4.6 Mutations of the Cyclic Nucleotide-Binding Domain (S672R, 695X) and of the C-Terminus (P883R, G1097W)

The CNBD domain is the hallmark of HCN and CNG (cyclic nucleotide-gated) channels and is also present in Erg/Eag channels, and its structural architecture is similar to that of the catabolite gene activator protein (CAP) of *E. coli* and to that of the cAMP binding site of the protein kinase A (Craven and Zagotta 2006; Biel et al. 2009). In HCN4 channels the CNBD extends from residue D600 to residue D712,

and binding of cAMP molecules to this site results in an allosteric facilitation of the voltage-dependent channel opening process. Following the CNBD, there is the proper C-terminus.

5.4.6.1 S672R

The heterozygous loss-of-function mutation p.S672R was identified by our group in bradycardic members of an Italian family. Mean heart rates calculated for mutation carriers was 52.2 bpm ($n = 15$), while those presenting a normal phenotype had a mean value of 73.2 bpm ($n = 12$). Given the large number of genetically related individuals investigated, it was possible to quantitatively assess the co-segregation between the phenotype (bradycardia) and genotype (p.S672R) and the presence of a tight linkage was confirmed by a LOD score value of 5.47. Sequence alignment analysis confirmed that the wild-type S672 residue is highly preserved in the phylogenetic tree from invertebrates to humans, an observation supporting its functional importance. Extensive electrophysiological investigation has shown that despite its localization within the CNBD, this mutation does not influence the affinity of the channel for the second messenger cAMP but rather renders the channel less sensitive to opening secondary to membrane hyperpolarization (V½: −76.1 mV and −84.5 mV for wild-type and homomeric mutant channels, respectively), and this action fully resembles that of the muscarinic modulation of the current.

5.4.6.2 695X

During a screening specifically aimed at the identification of common causative genetic defects leading to familial electromechanical disorders (sinus node disease and noncompaction cardiomyopathy), Schweizer et al. (2014) identified the novel mutations p.695X and p.P883R (in addition to the p.G482R previously described).

The mutation p.695X was found during a candidate gene study in genetically related heterozygous patients affected by sinus bradycardia and noncompaction cardiomyopathy (Schweizer et al. 2010, 2014). Because of the presence of an early stop codon, the truncated 695X protein lacks the CNBD, and expression studies in HEK 293 cells coherently showed that both homo- and heteromeric mutant channels were insensitive to cAMP-induced modulation. In addition, a rightward shift (+7.4 mV) of the half-activation voltage (V½) was observed in homomeric mutant channels.

Despite the lack of cAMP sensitivity of mutant channels, patients carrying the heterozygous 695X mutation exhibit normal chronotropic control and were able to reach normal maximal heart rates. In this respect it is worth noting that patients with the 573X mutation, which also removed the CNBD, were instead affected by chronotropic incompetence; the reason of this difference is unclear.

5.4.6.3 P883R

The missense mutation p.P883R was found in a single unrelated patient with sinus bradycardia, noncompaction cardiomyopathy, and paroxysmal atrial fibrillation; however, the phenotypic features of p.P833R mutant channels were never analyzed (Schweizer et al. 2014).

Patients carrying either the p.695X or the p.P833R exhibit the combined noncompaction cardiomyopathy and sinus bradycardia phenotypes.

5.4.6.4 G1097W

The mutation p.G1097W, located in the terminal part of the C-terminus, was found in a single patient with AV nodal dysfunction (AV block) but normal sinus node activity (Zhou et al. 2014). Upon heterologous expression, mutant current densities were significantly reduced and exhibit a negative shift of their voltage dependence (V½: wt, −86.6 mV; homomeric, −98.6 mV; and heteromeric, −94.2 mV). Modulation by cAMP was preserved. Since this mutation was found in a single patient and no sinus node alteration was observed, the genotypic-phenotypic association can only be hypothesized.

5.5 HCN Knockout and Transgenic Mice

The information obtained with in vitro single-cell experiments have provided a wealth of details on the importance of the cardiac pacemaker current; however, these studies lack the integration in the context of the entire organism. For this reason, different transgenic models have been developed to clarify the physiological contribution of HCN channels to cardiac pacemaking and heart development, as well as to improve our knowledge of their pathological relevance in arrhythmias. As discussed in the previous sections, the HCN4 isoform is the most functionally relevant in the mammalian SA Node, and its contribution to cardiac pacemaking and heart development has been studied by means of several HCN4 transgenic/KO mouse lines.

The first HCN4 knockout mice were developed by Stieber et al. (2003) who generated global and cardiac-specific constitutive HCN4 KO models by deleting the exon 4 which encodes for the pore region and the sixth transmembrane segment (TM6). Both global and cardiac-specific homozygous KO mice died between the embryonic days 9.5 and 11.5, but no structural abnormalities were noted in the developing heart. When isolated, the hearts of KO mice retained the intrinsic automaticity albeit they had a slower pace (−37% at day E9.5) than hearts isolated from wild-type embryos. Interestingly, action potential recordings carried out in single cardiomyocytes isolated from embryonic hearts of both models demonstrated the absence of a "mature" pacemaker phenotype which was instead observed in cells isolated from control wild-type embryo hearts. In agreement with these findings, HCN4 KO cardiomyocytes exhibit a 75–90% reduction of the I_f current, and the residual current could not be modulated by cAMP as opposed to a 10.3 mV positive shift of the activation curve observed for the wild-type I_f current. When the chronotropic behaviors of both single myocytes and whole hearts isolated from HCN4 KO mice were challenged with a membrane-permeable cAMP analog (8-Br-cAMP), no significant effects were observed. The finding that in the HCN4 KO constitutive models the I_f current and the spontaneous activities of both isolated hearts and cardiomyocytes are insensitive to the adrenergic mediator cAMP,

suggests that the I_f current represents a critical cAMP-sensitive element contributing to the chronotropic control of the heart at this developmental stage.

Although the lack of cAMP modulation might at first appeared as an additional "minor side effect" in comparison to the embryo lethality, its full relevance became clear a few years later when Harzheim et al. (2008) developed a cAMP-insensitive HCN4 knock-in (KI) transgenic model with a similar embryo lethality. The *HCN4* gene of this mouse was engineered to introduce the single amino acid exchange (R669Q) within the CNBD, and this mutation abolished the cAMP-induced modulation. Homozygous R669Q mice normally expressed the HCN4 protein, as verified by immunolabelling and Western blot experiments; however, they too died between embryonic days E11 and E12. Electrophysiological analysis confirmed that maximal f-channel conductance was similar in KI and wild-type cells, but the activation curve was shifted negatively by −13.2 mV in KI cells likely because of the lack of basal cAMP-induced modulation. In agreement with a reduced contribution of the I_f current in KI cells, automaticity of single cells and isolated heart was also reduced by 37% (at E9.5) and 40%, respectively. Experiments with isolated hearts also confirmed that neither adrenergic stimulation nor cAMP increase could enhance the beating rate of homozygous KI hearts.

Taken together the global, the cardiac specific, and the R669Q-KI constitutive KO models allow to conclude that the HCN4 current is necessary for proper cardiac embryo development and survival, and this crucial role appears to be related to the ability of the current to respond to cAMP/adrenergic stimulation. According to Harzheim et al. (2008), the need for a strong chronotropic response to catecholaminergic stimuli is indeed a vital aspect of the developing heart since it counteracts the otherwise life-threatening bradycardia which may occur during transient hypoxic states. However, a further element to consider is the evidence that the integrity of the HCN current is mandatory for the correct cell cycle progression of proliferating stem cells (Lau et al. 2011; Omelyanenko et al. 2016); whether this is an additional mechanism responsible for HCN4 KO embryo lethality is a question of remarkable interest.

Because of the lethality of KO embryos, the role of the pacemaker current during adulthood could not be evaluated by constitutive models. This limitation was soon overcome by Herrmann et al. (2007) and by Hoesl et al. (2008) who developed two inducible models, i.e. models where functional silencing of the *HCN4* gene could be obtained by knockout of the exon 4 (pore-S6 region) in a time-dependent manner. The two models differed in that the knockout was inducible and global in one case (Herrmann et al. 2007), while it was inducible but specifically restricted to all the cells of the organisms expressing the HCN4 channel in the other case (Hoesl et al. 2008). Despite the differences in the way they were engineered, the two models ultimately resulted in identical phenotypes, that is, the generation of non-functional HCN4 proteins in the entire organism. Single-cell studies reported that the I_f current density was indeed similarly reduced (by 75–80%) in both models, and 45–90% of SAN cells lacked spontaneous activity (Herrmann et al. 2007; Hoesl et al. 2008). In vivo recordings in adult mice showed that heart rate, ECG parameters, and chronotropic response to adrenergic stimulation were not different between control

and KO mice; however, recurrent sinus pauses and increased response to muscarinic stimulation were observed after KO induction. The presence of sinus pauses (which was also observed in isolated hearts) was maximal at heart rates of 350–450 bpm; of note is the observation that these rates (350–450 bpm) are slightly lower than the intrinsic heart rate of mice (Yaniv et al. 2016; Larson et al. 2013), and therefore sinus pauses are maximal in a condition where the vagal tone prevails. In line with this observation, the authors also report that in KO mice the bradycardic response to muscarinic stimulation was maintained, and pauses' duration was increased; on the contrary when the heart rate was raised by intense activity, therefore by robust sympathetic input, the number of sinus pauses decreased. These observations thus suggest that SAN cells of HCN4 KO mice have an "unstable" and "weak" diastolic pacemaker depolarization as a consequence of the paucity of the I_f current. This instability is clearly exacerbated by the parasympathetic-induced bradycardia, while it is partly resolved by the adrenergic action which increases the f-current. These results, which were partly unexpected giving the embryo lethality of the HCN KO constitutive models, suggest that in the adult mouse, additional mechanisms likely contribute to pacemaker generation and/or that the remaining I_f current after KO induction (20–25%) may be sufficient to drive the diastolic depolarization. Also relevant is the observation that while in the embryo heart the removal of the cAMP-dependent modulation of I_f impairs the chronotropic modulation, this is not the case in the adult where obviously other adrenergic modulatory mechanisms are at work.

As previously noted, the models by Hermann et al. (2007) and Hoesl et al. (2008) allowed to study the role of HCN4 channels in the adult animal; however, a possible confounding element of these models is that the knockout procedure occurred throughout all the cells of the organisms. Our laboratory therefore further improved the study model by creating a mouse line where the HCN4 knockout process was inducible and strictly restricted to cardiac cells (Baruscotti et al. 2011). This mouse thus presented the noticeable advantage of not altering the functional contribution of HCN4 channels in non-cardiac HCN4-expressing tissues (such as some types of neurons). This model exhibited a quite severe arrhythmic phenotype: severe bradycardia with a reduction of heart rate up to 47% (but sinus pauses were not observed), prolongation of the PQ interval, and AV block which progressed to complete AV block and heart arrest. SAN cells isolated from these mice consistently displayed a reduction of both spontaneous rate (up to ~60%) and I_f density (70%) highlighting therefore a solid cause-effect quantitative dependence between the reduction of the I_f current and SAN cells/cardiac rates (Fig. 5.3b).

In addition, despite the permanence of robust β-adrenergic chronotropic responses both in single SAN cells and in freely moving animals, the maximal rates attained were inferior to those elicited in wild-type conditions (cells, −43%; animals, −31%). We thus concluded that presence of an intact I_f current is required for proper cardiac impulse generation and modulation. While the association between HCN4 channels and SAN activity was obviously expected, our model provided the evidence for an active functional presence of HCN4 currents also in the AVN, suggesting an additional role of the HCN4 current associated with impulse conduction.

Alig et al. (2009) also developed a cardiac-specific and inducible HCN4 transgenic mouse model (Tet-Off system) based on the inducible and cardiac-specific expression of the human HCN4 isoform carrying the mutation 573X. This mutation, originally identified in a single patient by Schultze-Bahr, renders the HCN4 channel insensitive to cAMP modulation (Schulze-Bahr et al. 2003). The loss of cAMP modulation was indeed verified in single-cell experiments, since the I_f current recorded in SAN cells isolated from adult 573X mice was characterized by a negative shift of the voltage-dependent activation (~20 mV) and by the finding that isoprenaline could not increase the I_f current. Spontaneous activity of mutant cells was generally highly arrhythmic; however, a regular spontaneous AP rate could be restored in the presence of isoprenaline even though the maximal rate attainable was lower than that observed in wild-type cells. Adult mice expressing the HCN4X channels displayed a significant decrease of sinus rate both at rest and during exercise. Taken together these observations point to the conclusion that basal cAMP cellular content ensures a tonic control of the I_f current at rest and that an additional cAMP-dependent increase of the I_f can be recruited during adrenergic stimuli (i.e., during activity). This study shows that removal of cAMP-modulation during adulthood is not lethal, while embryo lethality was instead observed by Harzeim et al. (2008); despite this difference both models strongly provide the evidence that cAMP modulation of HCN4 channels is a key element to maintain proper basal heart rate as well as to provide a depolarization reserve that can be readily used upon the adrenergic stimulation and cytoplasmic cAMP increase.

The study of HCN4 transgenic models and the pathological role of HCN4 mutations underline the predominant role of this isoform during cardiac embryonic development and in the adult conduction tissue. However, since HCN1 and HCN2 expression have also been found in the human SAN, the cardiac phenotypes of these HCN1 and HCN2 KO models were also evaluated. Both HCN1 and HCN2 global and a cardiac-specific constitutive KO mice completed the embryonic development, indicating that these isoforms are not critical for cardiac development (Ludwig et al. 2003; Fenske et al. 2013; Nolan et al. 2003). Fenske et al. (2013) developed an inducible and global HCN1 KO mouse with sinus bradycardia, recurrent sinus pauses, increased heart rate variability, and lower maximal heart. Single-cell studies confirmed a strong reduction of the I_f current (~40%) and of both basal (−13%) and maximal (−13%) firing rates (Fenske et al. 2013). Somewhat similar results were observed by Ludwig et al. in adult HCN2 KO mice which exhibited sinus dysrhythmia which however disappeared in the presence of adrenergic stimulation (Ludwig et al. 2003). The I_f current measured in HCN2 KO SAN cells was ~30% less than in wild-type cells but was still modulated by cAMP (Ludwig et al. 2003). These data thus indicate that HCN2 channels provide a limited contribution to the adult pacemaker generation, and this contribution is mainly restricted to the basal rhythm. The physiological role of HCN3 isoform in the heart is still unclear; however, the HCN3 constitutive and global KO mouse developed by Fenske et al. (2011) did not show relevant phenotypic cardiac alteration in their chronotropic behavior.

Taken together the results obtained by the use of HCN transgenic mouse lines confirm the relevance of these channels in the control of cardiac rate. However, the

reader should be aware that given the obvious differences in cardiac rates between the murine models and the human heart, any attempt to interpret transgenic mouse data in the context of a unifying kinetic scheme of rhythm generation and control in humans may not be fully correct.

An inducible double HCN2/HCN4 knockout model restricted to working cardiomyocytes was developed by Hofmann et al. (2012) as a tool to study the mechanism underlying the arrhythmogenesis associated with cardiac hypertrophy and failure. Several studies had indeed previously demonstrated that the ventricular I_f current increases during these pathological states (Cerbai and Mugelli 2006). The study of Hofmann and colleagues (Hofmann et al. 2012) showed that even though HCN2 and HCN4 are the main isoforms expressed in the healthy ventricle, the isoform that is upregulated during cardiac hypertrophy is HCN1. Interestingly they also reported that when hypertrophy was induced after the knockout of the HCN2 and HCN4 isoforms, the risk of arrhythmogenesis was diminished.

5.6 HCN Channels: A Pharmacological Target for Therapy and Disease Treatment

The relevance of the I_f current in setting the slope of the diastolic depolarization of SAN cells makes it an important pharmacological target for selective modulation of heart rate. In the last few decades, different I_f blockers have been developed and extensively characterized by in vitro and in vivo studies (Bois et al. 1996; Monnet et al. 2001; Bucchi et al. 2002, 2006; Vilaine et al. 2003). These drugs, called "pure heart rate-lowering" agents, include alinidine (ST567), zatebradine (UL-FS49), cilobradine (DK_AH26), ZD-7288, and ivabradine (S16257). Apart from ivabradine, these drugs never reached the market due to the presence of undesired side effects such as block of cardiac K^+ and/or Ca^{2+} channels and block of neuronal HCN channels. Ivabradine is the only member of this family which caused minimal side effects (mild visual symptoms), and for this reason its efficacy has been extensively tested in three clinical trials developed to assess the beneficial effects of a selective reduction of heart rate in patients (1) with chronic heart failure with left ventricle systolic dysfunction [SHIFT trial (Swedberg et al. 2010)], (2) with both stable coronary artery disease and left ventricle systolic dysfunction [BEAUTIFUL trial (Fox et al. 2008)], and (3) with stable CAD without overt heart failure and left ventricle systolic dysfunction [SIGNIFY trial (Fox et al. 2014)]. These trials confirmed the effectiveness of ivabradine in relieving the symptoms of chronic stable *angina pectoris* in patients with coronary artery disease with normal sinus rhythm. The antianginal effect of ivabradine relays on its ability to selectively reduce heart rate; it is this rate reduction that improves oxygen supply to cardiomyocytes due both to an increase in the duration of the diastolic coronary perfusion time and to a reduction in cell oxygen consumption. Indeed, ivabradine reduced hospitalization of patients with stable, symptomatic chronic heart failure with reduced left ventricular ejection fraction ($\leq$35%) and in sinus rhythm with a resting heart rate $\geq$70 bpm. Ivabradine can be used as a single agent when β-blockers are not tolerated or

contraindicated or as add-on therapy when adequate heart rate control is not achieved. In contrast to the conventional pharmacological agents used to reduce heart rate (β-adrenergic blockers and calcium channel antagonists), ivabradine, at clinically approved doses, does not affect myocardial contractility, atrioventricular conduction, and hemodynamic parameters (DiFrancesco and Camm 2004; Sulfi and Timmis 2006). Finally, it is also worth noting that several case reports indicate beneficial effects of ivabradine for the treatment of inappropriate sinus tachycardia, postural orthostatic tachycardia syndrome, cardiogenic shock, and in case of uncontrolled heart rate following heart transplantation (Oliphant et al. 2016).

Ivabradine blocks native sinoatrial f-channels by entering from the intracellular side, and the resulting decrease in the pacemaker current causes a decrease of the slope of the diastolic depolarization of the action potential and thus of the heart automaticity (Fig. 5.4a, b) (Bois et al. 1996; Bucchi et al. 2002, 2007; Thollon et al. 1997).

An important feature of ivabradine block is its "use dependence" since the block is stronger when the channels repetitively cycle between the open and closed states, and this results in a block efficiency which is increased at high rates (tachycardia).

Investigation of the ivabradine binding site in HCN channels has highlighted that drug binding occurs in the water-filled cavity lined below the internal portion of the pore. In particular, the major determinant of ivabradine binding to the HCN4 channel is the structural integrity of the floor of the cavity in the closed state, which is formed by the side chains of the S6 residues Y506 and I510 (Bucchi et al. 2013).

Despite the existence of differences in the molecular details of the ivabradine block of HCN1 and HCN4 channels, the K_d values are similar (2.0 and 0.94 μM, for HCN4 and HCN1, respectively; from Bucchi et al. 2006), thus suggesting that

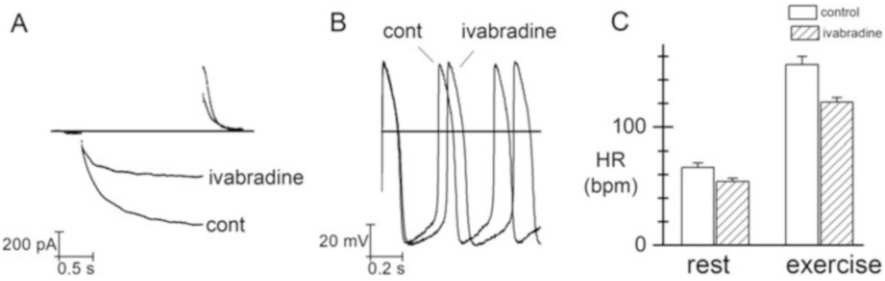

Fig. 5.4 Effect of ivabradine on native I_f, SAN Action potentials, and heart rate (HR). (**a**) Superimposed sample I_f current traces recorded from rabbit SAN cell in control condition and during ivabradine (3 μM) steady-state block. Currents were elicited by voltage steps at −100/+40 mV from a holding potential of −35 mV. Ivabradine reduced the I_f current by about 60%. (**b**) Superimposed sample AP traces recorded from rabbit SAN in control condition and following perfusion with ivabradine (3 μM). Ivabradine reduced the spontaneous AP rate by about 20%. (**c**) Effect of a single oral dose of ivabradine (30 mg) on the heart rate of healthy human volunteers at rest and during exercise (10 min exercise on a bicycle ergometer at increasing workloads). 2 hours after ivabradine intake, the heart rate was reduced by about 10% and 20% at rest and during exercise, respectively. Data shown in panel (**c**) are from Joannides et al. (2006)

ivabradine should not act as an isoform-selective drug. It is therefore this lack of selectivity that accounts for the partial block of HCN1 channels largely expressed in the retina that cause luminous phenomena/phosphenes which are mild side effects affecting vision (Cervetto et al. 2007; Demontis et al. 2009; Oliphant et al. 2016). It is thus important to develop a second-generation of isoform-selective blockers since this would allow to also target neurological conditions associated with dysfunctional neuronal HCN channels.

5.7 Conclusions

Since the discovery of the I_f current and the cloning of *HCN* channel genes a large amount of data have been collected on the properties and the physiopathological functions of this current using different models and approaches (including single cells, animal models, and newly published cryo-electron microscopy data on the structure of the human HCN1 channel). Although the cellular processes contributing, directly or indirectly, to pacemaker activity are many, the above data clearly indicate that the pacemaker current plays a substantial role in the generation and control of the SAN spontaneous activity. Evidence for this role includes (1) I_f activation range overlapping that of diastolic depolarization, (2) I_f-mediated control of cardiac rate by autonomic transmitters, (3) correlation between I_f expression in a given cell type and the presence of spontaneous activity, and (4) use of a specific f-channel blocker (ivabradine) as heart rate-reducing agent. HCN4 channel mutations also indicate a clear association between I_f and impulse generation, since most of the HCN4 dysfunctional mutations reported are associated with brady (loss-of-function) or tachy (gain-of-function) arrhythmias. In addition, experimental data also relate loss-of-function mutations with more complex pathologies such as AF, AV blocks, and structural abnormalities, thus suggesting new and still unexplored roles of HCN4 in the heart.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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