Control of Cardiac Rate by "Funny" Channels in Health and Disease

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Activation of the "funny" (pacemaker, I_f) current during the diastolic depolarization phase of an action potential is the main mechanism underlying spontaneous, rhythmic activity of cardiac pacemaker cells. In the past three decades, a wealth of evidence elucidating the function of the funny current in the generation and modulation of cardiac pacemaker activity has been gathered. The slope of early diastolic depolarization, and thus the heart rate, is controlled precisely by the degree of I_f activation during diastole. I_f is also accurately and rapidly modulated by changes of the cytosolic concentration of the second messenger cAMP, operated by the autonomous nervous system through β-adrenergic, mainly β_2, and in the opposite way by muscarinic receptor, stimulation. Recently, novel *in vivo* data, both in animal models and humans, have been collected that confirm the key role of I_f in pacemaking. In particular, an inheritable point mutation in the cyclic nucleotide-binding domain of human HCN4, the main hyperpolarization-activated cyclic nucleotide (HCN) isoform contributing to native funny channels of the sinoatrial node, was shown to be associated with sinus bradycardia in a large family. Because of their properties, funny channels have long been a major target of classical pharmacological research and are now target of innovative gene/cell-based therapeutic approaches aimed to exploit their function in cardiac rate control.

Key words: **pacemaker; funny current; I_f current; f-channels; heart rate; HCN channels**

Introduction

The physiological heart rhythm is generated in the sinoatrial node (SAN), a specialized region of the heart where myocytes spontaneously fire regular and repetitive action potentials, thus determining the rate of contraction of the whole heart. Spontaneous activity relies on the presence of the slow depolarization that develops during diastole and drives the membrane potential to firing threshold. Several mechanisms are involved in the generation of pacemaker activity; among them, a well-established role in initiating the slow diastolic depolarization is played by the pacemaker ("funny") I_f current.[1–4]

The pacemaker current was first described in the late 1970s[5] and named "funny" because its properties were, at the time, unusual in most respects when compared to those of all other known ion channels. Particularly unusual, I_f was found to be a mixed sodium/potassium inward current slowly activating on hyperpolarization below a threshold of about −40 to −45 mV.[6–8] I_f activation appeared therefore to be specifically relevant to generation of a slow depolarizing phase in the pacemaker range of voltages.

Another unusual feature of I_f is its dual activation by voltage and by cyclic nucleotides. Cyclic adenosine monophosphate (cAMP) molecules bind directly to f-channels and increase their open probability.[9,10] cAMP dependence is a particularly relevant physiological property, since it underlies the I_f-dependent autonomic regulation of heart rate.[5,8,11]

In the late 1990s, almost two decades after the first description of I_f, a family of genes encoding the molecular correlates of pacemaker channels was finally sequenced and cloned.[12–18] These genes and the corresponding protein products were named hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels; four isoforms are known in mammals (HCN1–4). Based on sequence similarities, HCN channels were shown to belong to the superfamily of the voltage-gated K^+ (Kv) and the cyclic nucleotide gated (CNG) channels. HCN channels are composed of six transmembrane domains (S1–6), with the positively charged S4 domain acting as the voltage sensor, a pore region, characterized by the GYG sequence typical of K^+-permeable channels, and a

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Ann. N.Y. Acad. Sci. 1123: 213–223 (2008).
doi: 10.1196/annals.1420.024

cyclic nucleotide binding domain (CNBD), homologous to that of CNG channels, at the C-terminus.

Expression of the various HCN isoforms in heterologous expression systems (human embryonic kidney [HEK] cells, Chinese hamster ovary cells, and *Xenopus* oocytes) elicits currents qualitatively similar to the native pacemaker current but quantitatively different in terms of voltage of activation, time constants, and sensitivity to cAMP. Activation/deactivation kinetics, for example, are fastest for HCN1 and slowest for HCN4, while HCN2 and HCN3 have intermediate activation/deactivation rates. Furthermore, HCN2, HCN3, and HCN4 channels are sensitive to cAMP, while HCN1 channels are nearly insensitive (for reviews, see Refs. 3, 4, 19, 20).

Of the four HCN isoforms, HCN4 is the most highly expressed in the SAN.[21,22] Although the embryologic origin of the cardiac conduction system and in particular of the SAN is far from being fully resolved, some of the steps leading to the development of the cardiac pacemaker region are understood. For example, it has been shown recently that the SAN develops from cardiac progenitors of the second heart field following activation of a specific gene program.[23–25] Among the early genes activated specifically in the cardiac region committed to become the SAN, at embryonic day E7.5, is the HCN4 gene; indeed HCN4 delineates specifically the primary pacemaker region in embryonic as well as in adult tissue.[22,25]

I_f–Modulation of the Cardiac Rate

I_f–Mediated Autonomic Modulation

The properties of I_f make it a central player not only in the generation of cardiac pacemaking but also in the cellular mechanisms underlying autonomic modulation of heart rate.[5,8,11,26,27] Fine modulation of rate relies on fine control of the steepness of the slow diastolic depolarization operated by autonomic stimuli. By activation of β-adrenergic (β_1 and β_2) and muscarinic M2 receptors, respectively, the sympathetic and parasympathetic neurotransmitters control the cytosolic concentration of the second messenger cAMP.

A major contribution to the autonomic control of rate is provided by the cAMP dependence of f-channels. Binding of cAMP molecules to the C terminus of pacemaker channels increases the probability of f-channel opening via a positively directed shift of the voltage dependence of the activation curve.[9] A reduced intracellular cAMP concentration gives rise to the opposite action, i.e., a negative shift of the activation curve and a reduction of open probability at any given voltage (FIG. 1A). Sympathetic/parasympathetic control of intracellular cAMP, therefore, induces, through cAMP-induced f-channel modulation, an increase/decrease of the net inward current during diastolic depolarization and a consequent increase/decrease of firing rate, respectively (FIG. 1B).

I_f Is Modulated Preferentially by β_2-Adrenergic Receptors

The adaptive function of sympathetic and parasympathetic innervation relies on the ability of the autonomic nervous system to respond effectively to rapidly changing physiological demands; accordingly, the signal transduction pathways leading to rate modulation need to be rapid.

At the cellular level, rapid and effective rate modulation is achieved through the contribution of two distinct processes. First, direct binding of cAMP to pacemaker channels guarantees a quicker and more specific modulation than that achieved by, for example, a cAMP-mediated phosphorylation process. Second, efficient modulation relies on the compartmentation of cAMP signaling.

cAMP is an intracellular second messenger activating several cellular processes, and its concentration is strictly controlled by cellular compartmentation of the biochemical factors responsible for cAMP synthesis and degradation, i.e., adenylyl cyclase and phosphodiesterases, in order to prevent unwanted spreading of cAMP signaling.[28–32] This requires that variations of cAMP occur in close proximity of the final target. We have shown[33,34] that pacemaker channels, and specifically HCN4 subunits, are confined, together with the β_2-adrenergic receptors (β_2-ARs), to membrane caveolae, cellular microdomains whose function is to keep in close proximity proteins involved in a specific signal transduction pathway.[35–39]

In cardiac cells, the physiological response to sympathetic stimulation is mediated by both β_1 and β_2 subtypes of β-ARs. In their expression patterns in cardiac cells, these two subtypes differ both in the density and localization. β_1-ARs are generally more abundant and widely distributed than β_2-ARs in the whole heart; in the SAN, however, β_2-ARs are expressed at a much higher level than in the rest of the heart.[40–44] Furthermore, whereas β_2-ARs are specifically localized in caveolae, β_1-ARs are for the most part excluded from these structures.[36,45,46] We have shown that in rabbit SAN myocytes, β_2-ARs co-localize with the HCN4 isoform of pacemaker channels in membrane caveolae.[34] As illustrated in FIGURE 1C and D, specific β_2 stimulation (obtained by combined β_2 stimulation with

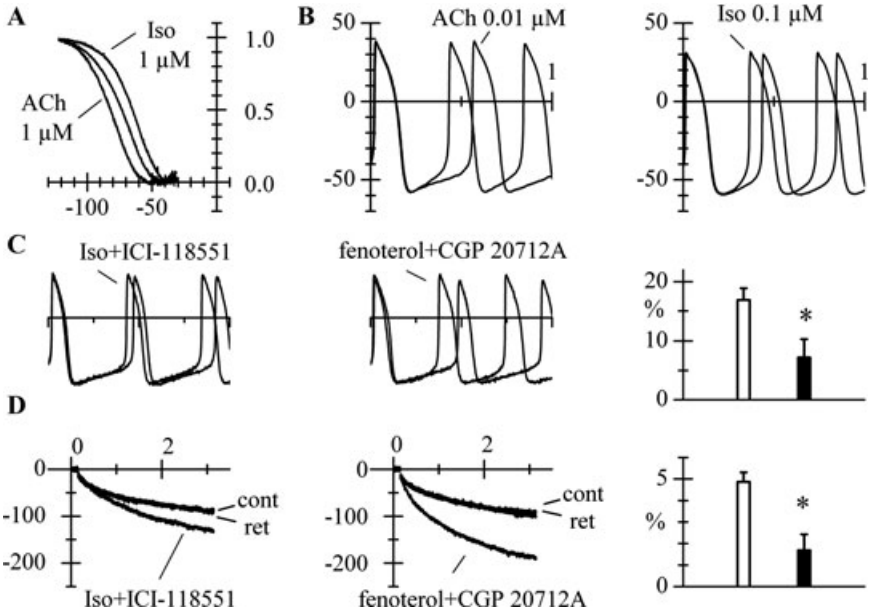

FIGURE 1. Funny channels mediate autonomic modulation of rate. **(A)** I_f activation curve is shifted negative/positive, which decreases/increases current, by muscarinic/β-adrenergic stimulation according to a mechanism involving cAMP-dependent channel modulation. **(B)** A decreased I_f reduces the steepness of diastolic depolarization and slows rate in the presence of ACh (*left*); the opposite occurs in the presence of Iso (*right*). **(C, D)** β_1-specific activation, obtained by perfusion with isoproterenol 1 μM + ICI-118551 10 μM, accelerates rate (7.2%; **C,** *filled bar*) and shifts the I_f activation curve (1.7 mV; **D,** *filled bar*) less than β_2-specific activation obtained by perfusion with fenoterol 10 μM + CGP20712A 0.3 μM (16.9%; **C,** *white bar*, versus 4.9 mV; **D,** *white bar*, respectively). (Data in **C** and **D** modified from Ref. 34 with permission).

fenoterol and β_1 inhibition by CGP 20712A) results in a larger shift of the I_f activation curve (4.9 mV) and stronger rate acceleration (16.9%) than does specific β_1 stimulation (obtained by combined β_1 stimulation by isoproterenol and β_2 inhibition by the specific β_2-inverse agonist (ICI-118551): 1.7 mV and 7.2%, respectively).[34] These data show the existence of a preferential β_2-mediated modulation of funny channels and control of cardiac chronotropism.

I_f Is Responsible for Rate Control during Vagal Tone

When I_f was first shown to be inhibited by ACh,[47] the general belief was that the mechanism responsible for rate slowing by vagal stimulation was the activation of an ACh-dependent K^+ current ($I_{K,ACh}$[48]). This view was challenged by the findings that inhibition of I_f occurs at 20-fold lower concentrations than activation of $I_{K,ACh}$, and that ACh concentrations able to inhibit I_f slow cardiac rate substantially.[11] The impact of this finding on the interpretation of the parasympathetic regulation of rate was twofold: first, these data showed for the first time that ACh-induced I_f inhibition, rather than K^+-current activation, is the process underlying slowing of cardiac rate by low ACh doses; second, evidence that both I_f and rate are sensitive to fairly low ACh concentrations introduced the novel concept that rate control during vagal tone is mediated by I_f. Thus, under resting conditions, a mildly inhibited I_f keeps rate to the normal, low resting level of about 70 bpm, and rate acceleration associated with removal of vagal tone, by pharmacological means, for example, is likely caused by removal of resting I_f inhibition.

Functional Relevance of I_f. Genetic Approach

Relevance of I_f to pacemaking is a long debated issue (for reviews, see Refs. 4, 8, 19, 49, 50). Cloning of the HCN channels has represented important progress in the understanding of the functional properties of I_f and has provided the basis for further experimental evidence supporting the role of funny channels in generation of spontaneous activity and rate control. Expression of HCN channels in the heart is high in the

conduction system, including the SAN, the atrioventricular node (AVN), and the Purkinje fibres.[2,3,8,51]

The possibility to specifically knock out a gene in mice has provided direct support for the functional role of HCN channels in heart rate control. HCN2-deficient mice display, along with various neuronal disturbances, alteration of sinus rhythm (sinus dysrhythmia), although rate modulation by autonomic neurotransmitters is normal.[52] Homozygous cardiac-specific HCN4-deficient mice ($HCN4^{-/-}$) die early during embryogenesis (between E9.5 and E11.5), and during their short embryonic life display a significantly reduced cardiac rate. Furthermore, $HCN4^{-/-}$ animals show a 75–95% reduction in I_f amplitude and are insensitive to isoproterenol-induced β-adrenergic stimulation.[53]

Taken together, these data indicate that HCN4 is necessary for the correct development of the cardiac pacemaker and is the major contributor to native SAN pacemaker current in mice; HCN4 is also the main mediator of β-adrenergic modulation. HCN2 is important for the correct propagation of electrical stimuli throughout the heart and contributes, even though less than HCN4, to I_f in mice but does not mediate β-adrenergic stimulation.

While transgenic animals have proved important in providing direct evidence for the involvement of HCN channels in the generation and modulation of cardiac rhythm, results highlighting the pacemaking role of HCN in humans have been slower to come. Early data investigating the existence of a link between mutations of the *hHCN4* gene and cardiac dysfunctions were either based on a single-patient report[54] or could not identify specific aspects of a complex arrhythmic behavior associated with a given mutation[55] and did not, therefore, provide compelling evidence for this link.

More recently, however, data showing that mutations of the *hHCN4* gene are indeed associated with specific rhythm disturbances have been obtained. In particular, an inherited mutation of a highly conserved residue in the CNBD of the HCN4 protein (S672R; FIG. 2A and B) was shown to be associated with inherited sinus bradycardia in a large Italian family spanning three generations.[56]

The 15 individuals heterozygous for the mutation had a significantly slower heart rate (mean rate: 52.2 bpm) than the 12 wild-type individuals (mean rate: 73.2 bpm). Heterologous expression in HEK293 cells of the mutated hHCN4 cDNA showed that the S672R mutation causes a change in the biophysical properties of HCN4 channels. Heteromeric wild-type/S672R channels had a more negative activation curve than wild-type channels (−4.9 mV shift) and faster deactivation kinetics, leading to reduced inward current during diastole and slower rhythm.[56] In its action, the mutation mimicked the effect of a low concentration of acetylcholine (about 20 nM) such as that released during mild vagal stimulation.[11] In FIGURE 2C, slowing induced by ACh 0.01 μM (−3.2 mV shift according to Ref. 11) is shown for comparison.

These findings confirmed the relevance of funny channels in the control of cardiac rate and provided novel evidence supporting the view that malfunctioning channels can cause disturbances of normal rhythm.

Pharmacological Approach

The involvement of funny channels in pacemaking makes them ideal targets for pharmacological interventions aimed to specifically modify heart rate. In the last two decades pharmaceutical companies have been actively searching for pure heart-rate-modulating agents, drugs able to change heart chronotropism without altering other parameters that might negatively influence cardiovascular performance (e.g., cardiac inotropism).

The concept of heart-rate reduction as a useful therapeutic intervention for some cardiovascular diseases is validated by several studies showing a tight association between elevated heart rate and mortality in subjects affected by coronary artery disease (CAD) as well as in the general population.[57] Many cardiovascular conditions such as chronic angina, ischemic heart disease, and heart failure are characterized by oxygen imbalance which would greatly benefit from a moderate reduction of heart rate. A lower basal heart rate indeed decreases cellular oxygen demand and improves myocardial perfusion by prolonging diastole.

Effective and widely used pharmacological interventions aimed to reduce heart rate are based on Ca^{2+}-antagonists and β-blockers. These compounds slow the heart efficiently, but have the undesired side effect of reducing cardiac inotropism due to the decreased contractile force developed by the myocardium when Ca^{2+} entry is reduced.

Starting in the 1980s, compounds able to reduce the steepness of the slow diastolic depolarization and slow cardiac rate with limited effects on cardiac inotropism were developed. These drugs were named originally "pure bradycardic agents" (PBAs) and subsequently "heart-rate reducing agents" after their properties (reviewed by Ref. 3), and were shown to exert their action by blocking pacemaker f-channels.[58,59]

f-channel blockers with heart-rate reducing action include alinidine (the N-allyl-derivative of clonidine),

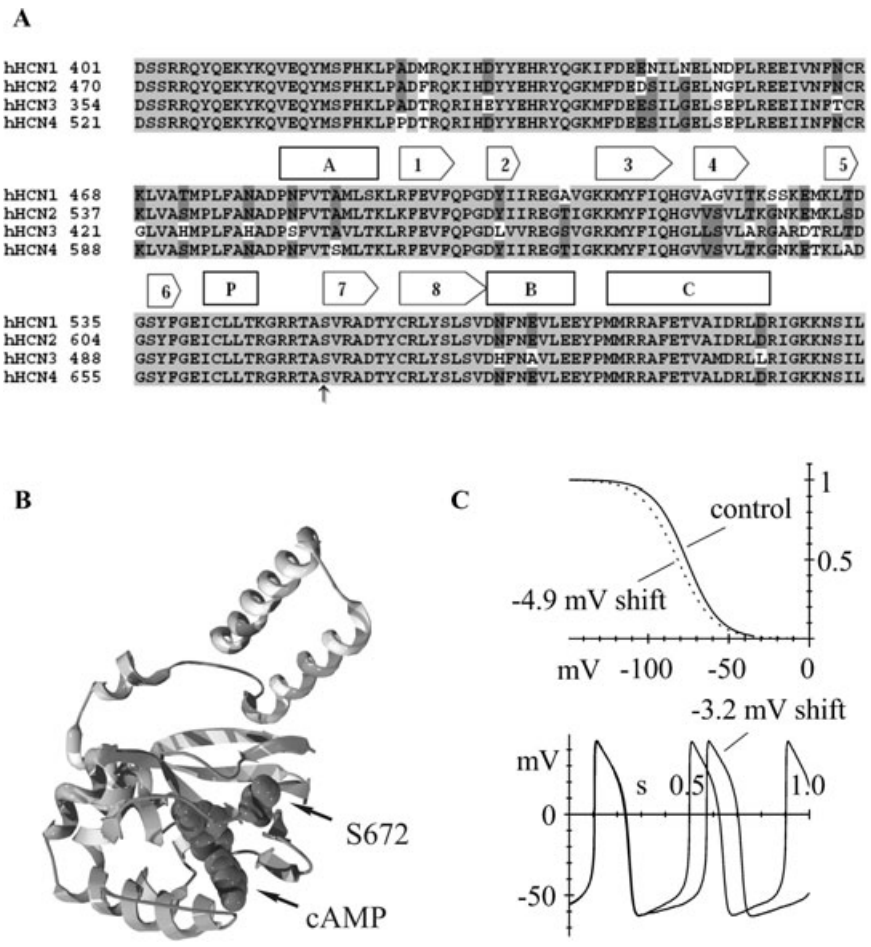

FIGURE 2. Single-point mutation of HCN4 associated with sinus bradycardia. **(A)** Sequences of the CNBDs of the four human HCN isoforms (1–4). Flat and arrowed rectangles represent α helices and β sheets, respectively. In the heterozygous mutation found in bradycardic individuals from a large Italian family,[56] a serine is replaced by an arginine (S672R in hHCN4, *arrow*). **(B)** Model 3D ribbon plot of the CNBD of hHCN4 obtained by DeepView-Swiss-PdbViewer homology modeling, based on the published mHCN2 CNBD crystal structure.[89] The mutated residue S672 is located close to the cAMP binding pocket: S672 and a bound cAMP molecule (*solid surface plots*) are indicated by *arrows*. **(C, top)** Relative to wild-type hHCN4 channels expressed in HEK293 cells (*full line*), heterozygous mutant + wild-type channels have a mean activation curve displaced to the left by 4.9 mV (*broken line*). (Data in **C, top,** modified from Ref. 56 with permission). **(C, bottom)** Slowing induced by ACh 0.01 μM, which causes a mean shift of −3.2 mV,[11] shown for comparison.

falipamil (AQ-A39), zatebradine (UL-FS49) and cilobradine (DK-AH26) (compounds originally derived from the Ca^{2+} channel blocker verapamil), and ZD7288. Unfortunately many of these molecules were shown to be potentially proarrhythmic, largely because at the concentration used to inhibit I_f they also affected other cardiac ion channels.[3,60,61]

Ivabradine is a more recently developed f-channel blocker and, to date is the only bradycardic agent validated and marketed for the treatment of chronic stable angina pectoris. Ivabradine was shown to slow the rate of isolated SAN cells by decreasing specifically the slope of the slow diastolic phase[62]; this effect could be attributed to a specific inhibition of I_f, since the T-type and L-type Ca^{2+} current and the delayed K^+ currents of SAN cells were unaffected at the same drug concentration (3 μM) blocking 60% of I_f.[63]

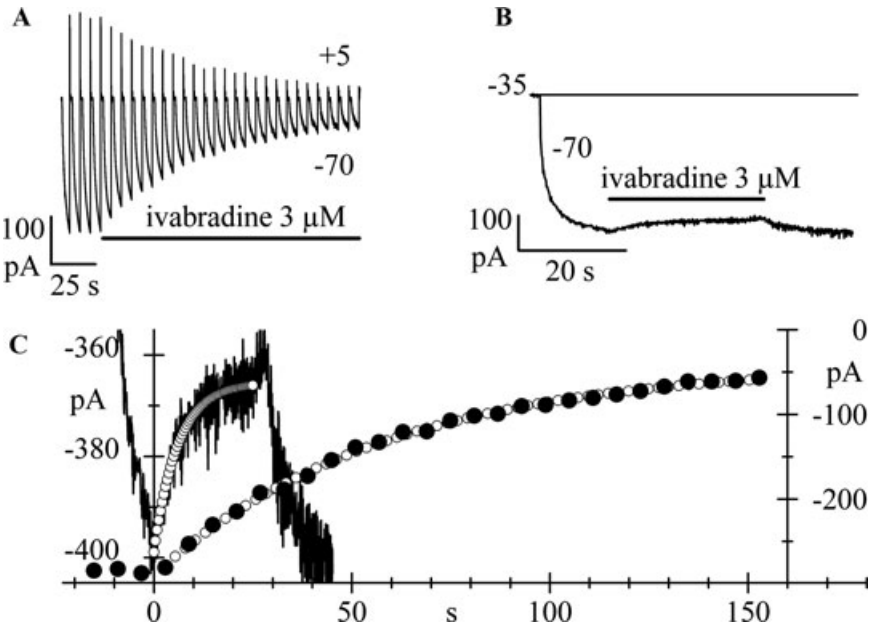

FIGURE 3. Block of If by ivabradine. **(A, B)** Pulsed protocol consisting of hyperpolarizing steps (4.5 s/−70 mV) followed by depolarizing steps (0.5 s/+5 mV), applied every 6 s from a holding potential of −35 mV **(A)** and long hyperpolarizing step to −70 mV **(B)**; application of ivabradine 3 μM results in a much larger block during application of the pulsed protocol than during the long step. **(C)** Time course of block development for the current at −70 mV from the experiments in **A** and **B;** fitting by single exponential decay (*open circles*) yielded steady-state blocks of 85.9% and 8.4% and time constants of 57.87 and 6.08 s for the pulsed protocol in **A** (*filled-circle curve*) and the long step in **B** (*noisy trace*), respectively.

The mechanisms of ivabradine block of native rabbit SAN f-channels and of individual HCN isoforms have been investigated in some detail.[64,65] In native f-channels, the drug acts by accessing the channel vestibule from the intracellular side and blocking the pore in a use-dependent and "current"-dependent way. Ivabradine is able to access its binding site only when channels are in the open state (state-dependence) and its block is favored by depolarization. As shown in FIGURE 3, ivabradine 3 μM applied during a pulsed protocol (activation at −70 mV, deactivation at +5 mV) induced a much larger block (FIG. 3A, about 86%) than that induced by a prolonged channel opening at −70 mV (FIG. 3B, about 8%). This behavior implies that block is strongly favored by depolarization. This voltage-dependent behavior can be shown to depend not so much on voltage itself, but rather by a "current"-dependence of block. In other words, when ions flow through the channel into the cell they tend to displace (kick-off) drug molecules from their binding site, while the opposite occurs in the short time when the current is outward during channel deactivation (kick-in).[64] Since channels open on hyperpolarization, there appears to be a contradiction between the need to hyperpolarize (in order to open channels) and to depolarize (in order for significant block to occur); on the contrary, however, these features are the basis for a strong "use dependence" of ivabradine action. This is an useful property: during tachycardia, for example, f-channels will undergo high-rate cycling between open and closed states, and the rate-reducing effect of ivabradine will be stronger. It is, however, interesting to notice that high efficiency of block during high-rate cycling between open and closed states is due to a larger degree of block at steady state, but is not associated with a faster onset of block. Surprisingly, block is weaker, but much faster during long hyperpolarizing steps than during pulsed cycling. In FIGURE 3C, for example, the time constant of block onset during hyperpolarization to −70 mV was 6.8 s, about 10-fold faster than that of 57.9 s during the pulsed protocol.

The action of ivabradine has also been investigated in individual HCN isoforms, with the aim of gaining information on the molecular mechanism of drug-channel interactions. A comparative analysis of cilobradine, zatebradine, and ivabradine blocking

effect has shown that all these compounds exert a use-dependent block on all HCN isoforms, with half-block values comparable to those found for native f-channels.[60,63,65]

Since HCN4 and HCN1 both contribute to native funny channels in rabbit SAN myocytes, the former being by far the most highly expressed isoform,[21,22,66,67] a detailed study has been performed of ivabradine block of these two HCN isoforms.[65] This study has highlighted important differences in the two block mechanisms. For example, while ivabradine behaves as an "open channel" blocker of HCN4 channels, as on native channels, it behaves as a "closed channel" blocker of HCN1 channels.[65]

Differences between blocking mechanisms of individual isoforms are especially relevant to the issue of specificity of drug action. A small fraction of patients under chronic use of ivabradine, for example, report visual disturbances (phosphenes), which are normally mild and can be reversed upon withdrawal from treatment.[57] These symptoms are attributable to the action of ivabradine on HCN channels expressed in the retina.[68] Since HCN1, but not HCN4, appears to be highly expressed in photoreceptors,[68] knowledge of the molecular basis for block of individual HCN isoforms can be exploited for designing new drugs with improved function and reduced side effects.

It is now recognized that heart rate reduction is an important factor in preventing ventricular remodeling and thus ejection impairment in conditions of chronic heart failure.[69] Specific inhibition of I_f can, therefore, be a valuable tool also for the treatment of patients with stable CAD and chronic heart failure. Two presently running large clinical trials, BEAUTIFUL and SHIFT, aim to evaluate the possible use of ivabradine in reduction of morbidity and mortality in CAD and heart failure patients.[70,71]

Biological Pacemakers

In the past decade, several laboratories have used both gene-based and cellular-based approaches to manipulate cellular processes involved in cardiac pacemaking. The intent of these manipulations was to generate, in normally quiescent substrates, stable rhythmic activity similar to that of native pacemaker myocytes. Gene-based strategies used so far to induce pacemaker activity followed different strategies including: 1) the upregulation of the β-adrenergic pathway[72,73]; 2) the downregulation of the resting potential stabilizing conductance I_{K1}[74]; and 3) the overexpression of pacemaker channels.[75–79]

In relation to the approach involving pacemaker channels, early attempts showed that overexpression of the HCN2 pacemaker channel induced a large increase in rate of spontaneously beating neonatal ventricular myocytes, mostly by steepening the diastolic depolarization.[75] Additional studies later confirmed that *in vivo* injection of adenovirus carrying the HCN2 gene into the left atrium or into the ventricular conduction system of dogs could induce a persistent spontaneous activity which, after sinus rhythm suppression by vagal stimulation, originated from the site of injection. Electrophysiological analysis demonstrated a significant increase of I_f density in cells isolated from the injected regions compared to control animals.[76,77,79]

Following these studies, other groups have proved that *in vivo* overexpression of HCN isoforms other than HCN2 were able to generate spontaneous activity in different substrates (HCN1[80]; HCN4[81]). These data indicate that the expression of pacemaker channels is sufficient to generate repetitive spontaneous activity in normally quiescent cells, in accordance with the established role of these channels in cardiac pacemaking.

Due to limitations in the duration of protein expression driven by adenoviruses and in the safety of adenoviral constructs, researchers have also explored the possibility of generating a biological pacemaker using a cell-based approach in which undifferentiated mesenchymal stem cells were engineered to express high levels of HCN2 channels.[78] This cellular substrate was able to influence, both *in vitro* and *in vivo*, the beating rate of an electrically coupled excitable substrate. It is interesting to note that mesenchymal stem cells overexpressing HCN2 are not pacing cells (in fact, they are not even excitable cells). The mechanism of pacing is based on the hypothesis that in the presence of electrical coupling, the membrane voltage of mesenchymal cells can be made to hyperpolarize to voltages where HCN2 channels are activated, which in turn will induce in the surrounding tissue a depolarization strong enough to drive pacemaker activity.[78]

By showing that overexpression of HCN channels is able to induce pacing, these strategies represent a successful "proof of concept." However, they also have drawbacks, such as 1) the limited duration of HCN expression, especially with the direct adenovirus-mediated infection; 2) the effect of HCN overexpression on ion concentration: in the long term, pacing driven by overexpression of HCN channels could result in cycle-length instability due to internal K^+ deprivation according to model computations[82]; and 3) lack of stability of undifferentiated mesenchymal stem cells, whose *in vivo* behavior is not predictable; indeed these cells have been shown to differentiate *in vitro*

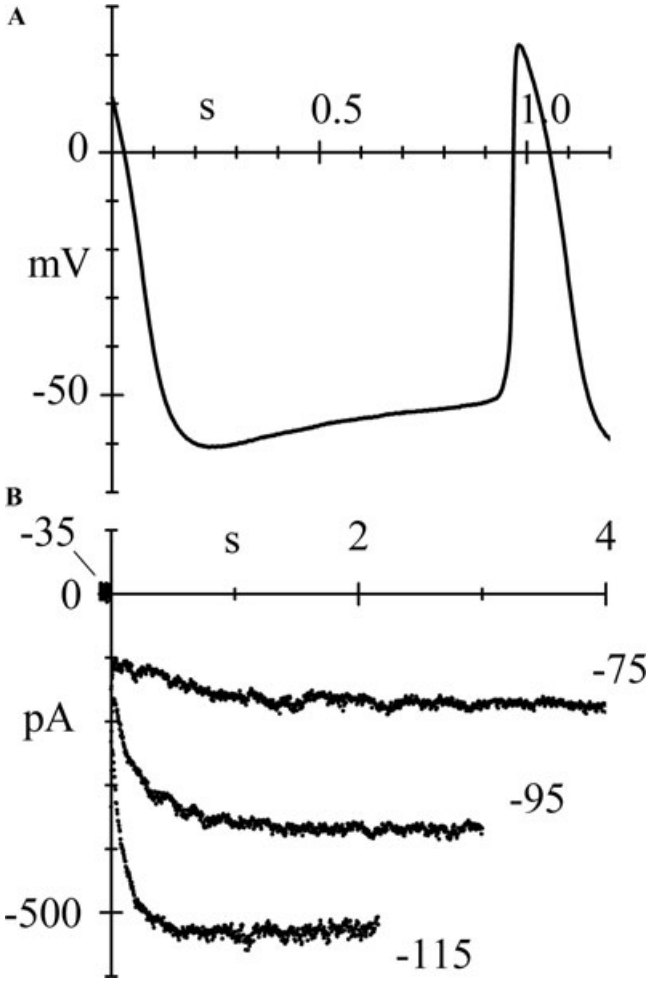

FIGURE 4. Activity recorded from ES cell-derived pacemaker myocytes. **(A)** Spontaneous action potentials. **(B)** I_f traces recorded during steps from a holding potential of −20 mV to the voltages indicated. Recordings were made at 36°C in normal Tyrode solution (see Ref. 34).

and *in vivo* toward several different phenotypes.[83] Differentiated cells could in principle undergo a remodeling process resulting in potentially arrhythmogenic alteration in the expression of HCN and other ion channels.

An alternative approach potentially able to prevent the drawbacks described above would be to use stem cell–derived or engineered cellular substrates with characteristics as close as possible to native pacemaker myocytes. So far, of the different types of stem cells investigated, only embryonic stem cells (ESCs) have been convincingly shown to differentiate into myocytes with a pacemaker phenotype.

Early results showed that murine ESCs can differentiate into cardiac myocytes by following a specific procedure. First, cells are expanded into aggregates called embryoid bodies (EBs), which include a portion with contractile cells; then, mechanical and enzymatic dissociation of the EB contractile portions leads to isolation of cells with action potentials typical of pacemaker cells and expressing the pacemaker I_f current.[84–86] In FIGURE 4 action potentials and I_f traces recorded from ESC-derived pacemaker myocytes with the above procedure (9 days after EBs plating on gelatin-coated dishes) are shown.

A cellular approach to biological pacemaking has been attempted using human embryonic stem cells (hESC) differentiated into spontaneously contracting EBs.[87,88] The contractile portion of an EB was shown to pace *in vitro* neonatal rat cardiac myocytes and *in vivo* the whole heart of either swine[87] or guinea pig.[88] Although these results were obtained with heterogeneous substrates and the molecular mechanism underlying pacemaking was not directly investigated, it is likely

that I_f played there an important role in the generation of rhythmic activity. Evidence for the contribution of I_f derives, for example, from the observation that perfusion of the f-channel blocker ZD7288 was able to slow rate,[88] and that β-adrenergic stimulation by isoproterenol was effective in increasing beating rate.[87,88]

Summary

The role of funny channel activation in the generation of rhythmic activity and control of heart rate has been consolidated through a substantial collection of data since the I_f current was originally identified and described nearly 30 years ago by Brown *et al.*,[5] and is now well established. Recent new applications of the concept of f-channel-based pacemaking have led to important developments of clinical relevance. These include: 1) a pharmacological application to heart-rate reduction therapies for the treatment of angina, achieved by specific f-channel inhibitors like ivabradine; 2) a genetic application to studies of inherited arrhythmias associated with mutations of human HCN4 subunits, such as the bradycardia-inducing S672R mutation; and 3) application to interventional medicine related to development of cell-based biological pacemakers potentially able to eventually replace electronic devices.

The clinical impact of f-channel-mediated pacing is likely to grow in the future in connection with the development of other, more detailed and more refined applicative tools.

Acknowledgments

This study was partially supported by European Union (Normacor) and CARIPLO (2004.1451/10.4878) grants to D.D.

Conflict of Interest

The authors declare no conflicts of interest.

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