The Role of the Funny Current in Pacemaker Activity

Dario DiFrancesco

Abstract: Pacemaking is a basic physiological process, and the cellular mechanisms involved in this function have always attracted the keen attention of investigators. The “funny” (I_f) current, originally described in sinoatrial node myocytes as an inward current activated on hyperpolarization to the diastolic range of voltages, has properties suitable for generating repetitive activity and for modulating spontaneous rate. The degree of activation of the funny current determines, at the end of an action potential, the steepness of phase 4 depolarization; hence, the frequency of action potential firing. Because I_f is controlled by intracellular cAMP and is thus activated and inhibited by β-adrenergic and muscarinic M2 receptor stimulation, respectively, it represents a basic physiological mechanism mediating autonomic regulation of heart rate. Given the complexity of the cellular processes involved in rhythmic activity, an exact quantification of the extent to which I_f and other mechanisms contribute to pacemaking is still a debated issue; nonetheless, a wealth of information collected since the current was first described more than 30 years ago clearly agrees to identify I_f as a major player in both generation of spontaneous activity and rate control. I_f-dependent pacemaking has recently advanced from a basic, physiologically relevant concept, as originally described, to a practical concept that has several potentially useful clinical applications and can be valuable in therapeutically relevant conditions. Typically, given their exclusive role in pacemaking, f-channels are ideal targets of drugs aiming to pharmacological control of cardiac rate. Molecules able to bind specifically to and block f-channels can thus be used as pharmacological tools for heart rate reduction with little or no adverse cardiovascular side effects. Indeed a selective f-channel inhibitor, ivabradine, is today commercially available as a tool in the treatment of stable chronic angina. Also, several loss-of-function mutations of HCN4 (hyperpolarization-activated, cyclic-nucleotide gated 4), the major constitutive subunit of f-channels in pacemaker cells, are known today to cause rhythm disturbances, such as for example inherited sinus bradycardia. Finally, gene- or cell-based methods for in situ delivery of f-channels to silent or defective cardiac muscle represent novel approaches for the development of biological pacemakers eventually able to replace electronic devices. (Circ Res. 2010;106:434-446.)

Key Words: pacemaking ▪ funny current ▪ rate modulation ▪ sinoatrial node ▪ HCN channels

Self-sustained contractile activity is a fundamental cardiac function, essential for life, and it is not surprising that its features have raised the interest of researchers since the earliest attempts at describing the anatomy and physiology of the heart. A realization of the presence of spontaneous activity can be found in the work of Claudius Galen, who in the second century AD observed that “the heart, removed from the thorax, can be seen to move for a considerable time . . . a
definite indication that it does not need the nerves to perform its function. Also, several centuries later, Leonardo da Vinci, who drew anatomic details of all organs of the body with unsurpassed draftsmanship and practiced his knowledge with dissected corpses, realized that the heart has autonomous activity. (“Del core. Questo si muove da sè, e non si ferma, se non eternamente”: “As to the heart: it moves itself, and doth never stop, except it be for eternity” [translated by Noble]).

A modern approach to understanding of pacemaker initiation and propagation had however to await a few more centuries, until the discovery of the bundle connecting atrium and ventricle by Wilhelm His, the atrioventricular node by Sunao Tawara, and the sinoatrial node by Arthur Keith and Martin Flack in the late 19th to early 20th century and the subsequent development of electrophysiological techniques. It is now well established that the sinoatrial node (SAN) is the natural pacemaker region of the heart, and that cells from this region underlie pacemaking. Unlike force-generating cells of the working muscle, whose main activity is mechanical, sinoatrial myocytes have the special property of self-generating repetitive action potentials and have correspondingly a poorly developed contractile system; their main function is electric, not mechanical. Action potentials originating from the central region of the sinoatrial node first propagate through the atria and then, after a slow transition across the atrioventricular node, spread through the ventricles via the specialized conduction tissue. SAN myocytes are therefore responsible for initiating spontaneous activity and controlling cardiac rate.

Given the basic relevance of pacemaking, the cellular/molecular processes underlying it have always been a major target of cardiac studies. What makes a SAN cell beat spontaneously? The original observation made by Galen on isolated hearts can be made today on single SAN cells, which keep beating after enzymatic isolation, as long as metabolic and environmental conditions permit; thus, although the SAN is the cardiac region most densely innervated by vagal and sympathetic nerve endings, generation of spontaneous action potentials is clearly an intrinsic property of SAN cells independent of innervation.

A distinguishing feature of spontaneously active cells, lacking in myocytes of the working muscle, is the presence in their action potential of the phase 4 diastolic (or pacemaker) depolarization. After termination of an action potential, during phase 4 the membrane voltage slowly depolarizes until it reaches threshold for firing of another action potential. Diastolic depolarization is therefore responsible for repetitive activity, and, to understand pacemaking, the interest of investigators has for obvious reasons concentrated on the mechanisms generating and controlling this phase of the action potential.

The pacemaker mechanism in heart, originally interpreted as resulting from the decay of an outward K⁺ current during diastolic depolarization, was re-evaluated in the late 70’s with the discovery in SAN cells of the “funny” current, i.e., an inward current activated on hyperpolarization in the diastolic range of voltages. This provided a new interpretation of pacemaking, according to which the pacemaker depolarization was generated by activation of the inward Iᵢ during diastole.

Several data collected during more than 30 years since its discovery have provided compelling evidence that the funny current has a major role in pacemaking and rate modulation, although this concept is still strongly debated in the light of the growing complexity of cellular processes involved in all aspects of rhythm generation and maintenance. A deeper understanding of the molecular basis of the properties of funny channels was achieved with the cloning in the late 1990s of their molecular correlates, the HCN (hyperpolarization-activated, cyclic-nucleotide–gated) channels. More recently, the interest in the concept of funny channel–based pacemaking has further grown thanks to the development of applications of this concept with a clinical relevance.

This review address the main properties of funny channels, their contribution to cardiac pacemaking and how the concept of funny channel-based pacemaking can be exploited in clinical applications. More detailed accounts of the biophysical properties of the molecular components of funny channels, the HCN channels, are given elsewhere.

**Physiological Evidence: Iᵢ Properties Are Right for a Mechanism in Charge of Generating a Slow (Diastolic) Depolarization Process and Modulate Its Rate**

**General Properties**

Details of the general properties of the funny current have been described in early and more recent review work and only essential features, and their physiological relevance are discussed here.

Iᵢ was originally described in pacemaker SAN tissue. It is activated on hyperpolarization at voltages below about −40/−45 mV and is inward in its activation range, its reversal potential being about −10/−20 mV, a result of the mixed Na⁺ and K⁺ ionic permeability of the current later investigated. Because activation of an inward current leads to depolarization, the simple observation that the Iᵢ activation range overlapped the range of voltages where diastolic depolarization occurs in SAN cells (approximately −40 to −65 mV) was by itself supportive of the notion that Iᵢ could be a suitable candidate for generating the diastolic phase, acting therefore as a “pacemaker” current. Further reinforcing this view was evidence that Iᵢ increases during perfusion with adrenaline. Iᵢ therefore appeared to have properties suitable not only for generation of spontaneous activity but also for sympathetic acceleration of rate.

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>AR</td>
<td>adrenergic receptor</td>
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<tr>
<td>HCN</td>
<td>hyperpolarization-activated, cyclic-nucleotide–gated</td>
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<td>SAN</td>
<td>sinoatrial node</td>
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**I\textsubscript{K2} Interpretation**

The discovery of $I_f$ represented the proposal of a novel mechanism, the activation of an inward current, able to explain generation of spontaneous activity, but also posed a problem, because this mechanism contrasted with a well established, apparently incontrovertible mechanism proposed more than a decade earlier to underlie pacemaker activity of Purkinje fibers: the decay of a depolarization-activated outward $K^+$ current, the $I_{K1}$ current.\(^5\) The $I_{K2}$-decay hypothesis was also supported by evidence for a $\beta$ adrenergic-dependent stimulation of the current able to contribute to sympathetic rate modulation.\(^{21}\) How was it possible that 2 completely different processes, based on currents with totally different ionic nature, could underlie pacemaker generation in 2 types of myocytes belonging to the same cardiac conduction system? The puzzle was even more baffling because to a close analysis, several features of $I_f$ in SAN cells appeared to be surprisingly similar to those of $I_{K2}$ in Purkinje fibers.\(^{22}\)

Two years after the original description of $I_f$, the puzzle was solved with the reinterpretation of the ionic nature and kinetics of the $I_{K2}$ current\(^6\)-\(^{20}\): $I_{K2}$ was in fact shown to be, like $I_f$, an inward current activated on hyperpolarization and carried by Na\(^+\) and K\(^+\), rather than a pure K\(^+\) current activated on depolarization. How an inward current like $I_f$ could behave like a pure K\(^+\) current, with a reversal potential close to the expected K\(^+\) equilibrium potential, was explained with the presence in Purkinje fibers of a large K\(^+\) inwardly rectifying component ($I_{K1}$), which decreased during the strong hyperpolarizing steps used to study $I_{K2}$;\(^{23}\) the superimposition of this component with $I_f$ generated a “fake” reversal potential close to the K\(^+\) equilibrium potential.

Note that SAN myocytes do not express an $I_{K1}$ component,\(^{24}\) a property responsible for the depolarized level of diastolic depolarization in these cells; thus, an $I_{K1}$-dependent K\(^+\) depletion process does not occur during voltage-clamp hyperpolarization, which explains why the inward nature of $I_f$ could be promptly identified in SAN cells, but was missed in Purkinje fibers.

The use of barium to block $I_{K1}$, and thus remove the $I_{K1}$-dependent component, transformed the $I_{K2}$ of the Purkinje fiber into $I_f$.\(^{6}\) This result was particularly dramatic because it unmasked the inward hyperpolarization-activated nature of $I_{K2}$, an apparently pure K\(^+\) current.

The identity between the 2 “pacemaker” currents in the 2 cardiac tissues led to an integrated theory for the origin of cardiac pacemaking in different pacing regions of the heart. A systematic characterization of the funny current followed the reinterpretation of $I_{K2}$ and its identification with $I_f$.\(^{2,25}\)

**Numeric Modeling**

Shortly later the first $I_f$ description and the reinterpretation of $I_{K2}$, the novel $I_f$ properties were integrated into numeric models and shown to be able to fully explain several aspects of SAN\(^{26}\) and Purkinje fiber\(^{16}\) electric activity, including the generation of diastolic depolarization and spontaneous action potentials.

The $I_f$-dependent contribution to the diastolic depolarization can be described simply on the basis of the $I_f$ properties. Briefly, when during the depolarized part of the action potential, at positive voltages, $I_f$ is completely turned off, no contribution is available. During repolarization however, when the voltage hyperpolarizes below about $-40/-45$ mV (the $I_f$ activation threshold), the current switches on and progressively increases, first opposing to and then stopping the repolarization process (at the maximum diastolic potential) and finally initiating the diastolic depolarization. Action potential repolarization is driven in rabbit SAN mostly by delayed K\(^+\) current ($I_{K1}$), the rapid delayed K\(^+\) current flowing through HERG channels,\(^{27}\) and during the last part of repolarization and the very early part of diastolic depolarization the net current will be composed primarily by a nearly equilibrated (slightly inward) sum of the inward $I_f$ and the outward $I_{K4}$; changes of $I_{K2}$ will therefore clearly be reflected by changes in the very early fraction of pacemaker depolarization, as well as during repolarization.

The $I_f$ contribution terminates when, in the late part of diastolic depolarization, Ca\(^{2+}\)-dependent processes take over and the threshold for L-type Ca\(^{2+}\) current activation and action potential firing is reached. Although deactivation of $I_f$ at depolarized voltages is rapid, complete switch off of the current will only occur during the very early fraction of the action potential, which provides a brief time interval during which $I_f$ carries an outward current at positive voltages. As is discussed below (see Figure 4), this time interval is essential for the action of specific f-channel blockers.

**Autonomic Rate Modulation**

The SAN of mammals is densely innervated with autonomic nervous fibers, regulating cardiac chronotropism. Sympathetic $\beta$-adrenergic stimulation acts by accelerating, and parasympathetic muscarinic stimulation by slowing cardiac rate. As mentioned above, the original description of $I_f$ in the SAN was accompanied by the finding that the current is also increased by adrenaline, in a way that could explain the accelerating action of sympathetic stimulation.\(^19\)

When SAN cells are superfused with solutions containing low concentrations of adrenergic agonists, acceleration of spontaneous rate is associated with a steeper slope of diastolic depolarization, with little modification of action potential duration and shape. A post hoc analysis of data from Brown et al (1979)\(^19\) illustrates this observation for their original records, as shown in Figure 1A (right), where the trace recorded in adrenaline is shifted so as to have superimposition of control and adrenaline action potentials.

Clearly, the adrenaline-induced rate acceleration is almost entirely attributable to the shortening of the diastolic duration associated with a faster slope of diastolic depolarization, whereas only minimal changes occur in the action potential shape and duration. This agrees with the hypothesis that the process responsible for diastolic depolarization (ie, $I_f$ activation) is an important target of sympathetic rate regulation.

Although the description of the $I_f$ response to adrenaline was a first indication of the involvement of $I_f$ in autonomic modulation of heart rate, much work was still needed to define more fully other aspects of the $I_f$ function. Several additional observations provided detailed description of $I_f$ features and more evidence supporting its relevance to pacemaker generation and rate control. It was shown for
example that β-adrenergic receptor (AR) stimulation increases \( I_f \) by shifting the activation curve of the current to more positive voltages, without modification of the conductance, a result also confirmed by single-channel measurements.\(^28,29\) The depolarizing shift of \( I_f \) activation curve is attributable to β-AR-dependent increase of intracellular cAMP, the second messenger in \( I_f \) modulation. As was later shown by macropatch analysis, cAMP positively shifts the \( f \)-channel activation curve not by a phosphorylation-dependent process, but directly by binding to channels.\(^30\) This was the first evidence, later confirmed with the cloning of HCN channels, that funny and CNG (cyclic-nucleotide gated) channels have similar properties and in fact belong to the same superfamily.

A more complete understanding of the \( I_f \) role in heart rate control was achieved in the mid/late 1980s, with the demonstration that \( I_f \) is strongly inhibited by parasympathetic stimulation, according to a mechanism opposite to that associated with β-AR stimulation, ie, a negative shift of the current activation curve attributable to muscarinic-induced inhibition of adenylyl-cyclase and cAMP reduction.\(^31,32\)

Vagal stimulation induces a negative chronotropic effect by releasing acetylcholine (ACh) (Figure 1B), and when the muscarinic modulation of \( I_f \) was discovered, the established view, based on early experiments,\(^33\) was that the mechanism responsible for ACh-induced slowing is the activation of an ACh-dependent \( K^+ \) current.\(^34\) The new evidence challenged this view and raised the question whether, and to what extent, the muscarinic-dependent \( I_f \) inhibition was involved in the vagal-induced negative chronotropism; why should 2 different mechanisms operate simultaneously to slow cardiac rate on vagal stimulation? This question was addressed by investigating the ranges of ACh concentration required to activate the 2 mechanisms. The result was surprising, in that the concentrations were quite different: whereas low doses of ACh (up to 0.01 to 0.03 μmol/L) were shown to inhibit \( I_f \), 20-fold higher concentrations were necessary to activate the \( K^+ \) current conductance; also, the low doses of ACh active on \( I_f \) inhibition (but not on \( I_{K,ACH} \) activation) were perfectly capable of slowing the rate of spontaneous activity of pacemaker cells (Figure 1B).\(^35\) The consequence of this finding was the introduction of a novel concept in the physiology of autonomic heart rate regulation: these results showed that the negative chronotropic effect of low-to-moderate vagal stimuli is mediated by \( I_f \) inhibition, not by activation of a \( K^+ \) current.

Along with the cAMP-dependent modulation, other mechanisms control the function of funny channels, as shown by studies of native channels and/or individual HCN isoforms; these include accessory subunits such as MiRP1,\(^36,37\) membrane phospholipids such as PIP2 (phosphatidylinositol 4,5-bisphosphate),\(^38\) and mechanisms affecting compartmentation of channels into membrane lipid rafts.\(^39\)

Phosphorylation by serine/threonine kinases (p38 mitogen-activated protein kinase)\(^40\) and by tyrosine kinases (Src) also contributes to regulate funny channel activity. The action of Src tyrosine kinase has been characterized with some detail. Phosphorylation by Src tyrosine kinase of residues Y476 (of mHCN2) or the analogous Y554 (of hHCN4), for example, was found to induce acceleration of channel activation and shift to more positive voltages the current activation curve.\(^41,42\) Specific screening of tyrosine residues of hHCN4 involved in Src-dependent phosphorylation further revealed that a major role in mediating the Src action is played by the tyrosine residue Y531.\(^43\) Src-dependent phosphorylation involves a sequence of HCN2 comprising part of the C-linker and the cyclic nucleotide binding domain, which might suggest an interaction between the actions of cAMP and phosphorylation; however, cAMP-dependent modulation is essentially preserved when Src phosphorylation is inhibited, except for a slowing of activation kinetics at saturating cAMP concentrations.\(^41\) Thus, cAMP-dependent and phosphorylation-dependent control of HCN2 appear to operate mostly through separate mechanisms.

**Rate Modulation Mediated by \( I_f \) Involves the Rate of Diastolic Depolarization and Does Not Affect Firing Threshold**

As much as low/moderate β-AR stimulation accelerates rate by promoting a steepening of diastolic depolarization, with little alteration of action potential shape and duration, so low/moderate muscarinic stimulation slows rate by a symmetrical action, ie, by decreasing diastolic depolarization rate without modification of action potential parameters (Figure 1). Detailed measurements show that neither sympathetic nor parasympathetic stimuli (when low/moderate) modify the action potential threshold.\(^44\)

The specificity of action on the steepness of diastolic depolarization implies that the autonomic transmitters act mostly on the process generating and controlling this phase. Thus, showing that a specific change in \( I_f \) modifies only the diastolic rate would represent strong evidence that the dia-
stolic depolarization is controlled by $I_t$ activation, and that autonomic rate modulation is mediated mainly by $I_t$. Indeed, block of funny channels by “pure heart rate-reducing” agents like ivabradine (at concentrations at which they can be considered as selective) can be shown to induce slowing by reducing the diastolic depolarization rate, with little alteration of action potential duration; this subject is treated more extensively below (see the section Pharmacological Evidence).

Spontaneous activity of pacemaker cells slows down also in the presence of ryanodine, whose action involves the emptying of Ca\(^{2+}\) stores and inhibition of SR Ca\(^{2+}\) transients; this leads to the suggestion that Ca\(^{2+}\) cycling may represent a main mechanism for rate regulation. Changes induced by SR Ca\(^{2+}\) transients inhibition on action potential configuration are however quite different from those attributable to muscarinic stimulation: the early fraction of diastolic depolarization remains unaltered, and prolongation of the late fraction is associated with a substantial shift to more positive voltages of the threshold of action potential firing, a change not observed on $I_t$ inhibition by ivabradine or vagal-induced slowing.\(^7,44\) These data indicate that autonomic control of chronotropism is performed via $I_t$ modulation, not modulation of Ca\(^{2+}\) transients.\(^7,44\)

The major role of $I_t$ in the control of rate and its autonomic modulation at low/moderate agonist concentrations does not exclude the involvement of other components, particularly in the late fraction of diastolic depolarization, when the contribution of Ca\(^{2+}\) currents and the Na\(^+/\)Ca\(^{2+}\) exchange current becomes more significant. It is worth noting in this respect that although the L-type Ca\(^{2+}\) current ($I_{Ca,L}$) has a much lower sensitivity to ACh than $I_t$, with EC50 values differing by orders of magnitude, the sensitivity to β-AR stimulation of the 2 currents is similar\(^45\); this suggests that although $I_{Ca,L}$ does not substantially contribute to parasympathetic rate control, it may contribute, along with $I_t$, to accelerate spontaneous frequency during β-AR stimulation.

However, data concerning the contribution of $I_{Ca,L}$ to rate control are variable. Block of $I_{Ca,L}$ by nifedipine can lead to slowing of diastolic depolarization,\(^46\) but if applied to peripheral nodal cells, nifedipine can lead to acceleration;\(^47\) also, action potential-clamp measurements show that, contrary to expectation, the nifedipine-sensitive current is outward during diastolic depolarization, possibly because of the presence of a Ca\(^{2+}\)-dependent K\(^+\) conductance.\(^48\)

A complicating factor in the analysis of the contribution of $I_{Ca,L}$ to rate control is that changes of $I_{Ca,L}$ normally modify action potential shape and duration and thus affect indirectly other components during activity. By acting specifically on the early fraction of diastolic depolarization without substantial alteration of action potential shape and duration (Figure 1), the funny current maintains a major, independent role in autonomic modulation of rate at low/moderate agonist concentrations.

**Why Does Inhibition of Ca\(^{2+}\) Transients (by Ryanodine) Impair β-AR Rate Modulation?**

According to the “Ca\(^{2+}\)-clock” hypothesis, pacemaking is regulated by the periodicity of Ca\(^{2+}\) transients, rather than by $I_t$.\(^49\) Evidence supporting this role includes data indicating that alterations of Ca\(^{2+}\) cycling are reflected by changes of spontaneous frequency and rate control. It is known for example that β-AR modulation of rate is strongly inhibited in cells where exposure to ryanodine has reduced or abolished Ca\(^{2+}\) transients.\(^50,51\) However, it has been shown that although reducing SR Ca\(^{2+}\) transients does impair β-AR rate modulation, it does not affect the rate acceleration caused by increasing intracellular cAMP levels.\(^52\) This suggests that disruption of proper Ca\(^{2+}\) homeostasis impairs one or more of the steps leading from β-AR stimulation to rate acceleration, but is not directly involved in the cAMP/f channel-dependent mechanism of rate regulation.

Such an action could be performed for example by a Ca\(^{2+}\)-dependent element in the cAMP signaling cascade, such as a Ca\(^{2+}\)-activated adenylate-cyclase. As predicted,\(^53\) a Ca\(^{2+}\)-dependent, “neuronal” form of adenylate-cyclase has indeed been recently shown to be expressed in the SAN but not in the ventricle of guinea pigs.\(^53\) The finding of a Ca\(^{2+}\)-activated adenylate-cyclase is particularly interesting because, in addition to explaining lack of β-AR–induced rate modulation after abolishment of SR Ca\(^{2+}\) transients, it also provides a basis to explain early data indicating a dependence of $I_t$ on intracellular Ca\(^{2+}\).\(^54\) although inside-out patch clamp data clearly indicated lack of a direct Ca\(^{2+}\) effect on f-channels.\(^55\) Ca\(^{2+}\) dependence of funny channels may also provide an interesting additional perspective to interpret data showing that elements involved in Ca\(^{2+}\) homeostasis (such as CaMKII [Ca\(^{2+}\)/calmodulin-dependent protein kinase II]) participate in autonomic rate regulation.\(^56\)

**HCN Channels As Basic Components of Biopacemakers**

When not responding to pharmacological treatment, life-threatening rhythm disorders such as symptomatic bradycardia, sick-sinus syndrome, atrioventricular nodal block and heart block often require the implantation of artificial pacemakers. Although electronic pacemakers are certainly effective in their function, they also have several drawbacks including lack of autonomic-driven modulation, need for a battery, etc, and in the last several years the development of biological pacemakers, potentially able to overcome these flaws, has attracted the interest of several laboratories.

Various approaches have been attempted. Gene-based methods include overexpression of β2-adrenergic receptors\(^57,58\) or of HCN channels\(^59–64\) and downregulation of K\(^+\) currents;\(^65\) cell-based methods involve the transfer of spontaneously beating cardiomyocytes derived from various types of stem cells.\(^66,67\)

Because funny channels have a specific role in pacemaking and do not directly affect phases of the action potential other than the diastolic depolarization, HCN-based biological pacemakers appear to be the basis for a potentially successful approach. HCN transfer has indeed proven successful in accelerating diastolic depolarization and spontaneous rate in proof-of-principle experiments where the HCN2 isoform was overexpressed in primary cultures of neonatal ventricular myocytes.\(^69\) Protocols investigated for development of HCN-based biological pacemakers include adenoviral-mediated HCN infection,\(^60,61,63,68\) chemically induced fusion of fibro-
blasts expressing HCN1 channels, and the use of mesenchymal stem cells engineered to overexpress HCN channels. Spontaneously beating human embryonic stem cell–derived cardiomyocytes have also been shown to integrate with and pace cultures of cardiac myocytes and whole hearts in vivo; it is known that embryonic stem cell–derived spontaneously active myocytes express \( I_f \).

As a whole, these results show that the transfer of HCN channel gene or of HCN-expressing myocytes is capable under proper conditions to induce in vivo pacing.

**Morphological Evidence: HCN4 Is a Marker of Pacemaker Tissue**

Since the original description by Keith and Flack in 1907, the SAN is recognized as the cardiac region where pacemaker activity is initiated. Understandably, the most complete set of experimental data presently available from the literature was collected from laboratory animals, and particularly from the rabbit, although recent investigation has allowed to identify the expression patterns of several ion channels also in the human sinus node, at both the mRNA and protein level.

Morphological and histological studies of the SAN show that this is a highly specialized region with features that favor the function of pacemaker generation and propagation to surrounding areas. Pacemaker cells have a poorly developed contractile apparatus, because their function is electric rather than mechanical, with little anisotropy so as not to favor a specific direction of propagation, although cell distribution and orientation appear to favor, if only to a limited extent, signal propagation in the direction parallel to the crista terminalis. Cell-to-cell coupling is poor in the SAN, which is mainly attributable to reduced density of the connexins specifically expressed in this region, typically connexin45 and connexin40 in the rabbit, dog and human SAN. A poor electric connection in the center of the node complemented by a progressively increasing intracellular coupling at the border with atrial muscle is thought to be functional to proper pacemaker generation, because it favors exit of the signal from the node center, limiting at the same time node hyperpolarization by atrial muscle.

Low electric conductivity is also attributable to a paucity of excitable cells relative to connective tissue, which represents approximately 50% of whole tissue in the rabbit and 45% to 75% in the human SAN. In the rabbit, nodal myocytes are organized in nonhomogeneous clusters interconnected by thin cytoplasmic extensions forming a 3D mesh-like structure. This feature may be species-dependent, because in contrast with the results in the rabbit, a region characterized by a compact and almost exclusive presence of nodal myocytes has been reported in the mouse SAN.

According to electrophysiological recordings from the mammalian SAN, the leading pacemaker site lies roughly in the area extending between the 2 venae cavae and adjacent to the crista terminalis. Recent investigation of the distribution of expression of HCN4 in rabbit SAN has shown that HCN4 proteins are highly concentrated in the central part of the node, corresponding to the leading pacemaker site, and that their density is progressively reduced when moving to more peripheral areas (Figure 2). Reliability of expression data were confirmed by verification that mRNA and protein expression patterns were overlapping (Figure 2, left and right, respectively). No detectable immunofluorescence was found in the surrounding crista terminals and septum interatrialis, confirming specific localization of pacemaker channels in the central node. Also, HCN4 membrane expression and \( I_f \) amplitude were correlated in cells isolated from crista terminals, central SAN area, and septum interatrialis. Sample current traces recorded during steps to \(-125\) (holding potential of \(-35\) mV) from single cells enzymatically dissociated from the regions indicated. Data adapted from Brioschi et al.

Figure 2. HCN4 is expressed in pacing cells of the SAN region but not in atrial cells and \( I_f \) density correlates with degree of HCN4 expression. A, Sections of rabbit SAN tissue made by cutting according to a grid perpendicular to the crista terminalis from the superior to the inferior vena cava, with grid interval of 2 mm (top to bottom). In all slices, the crista terminal is on the left and the interatrial septum on the right, and the endocardial surface faces up. Left images, HCN4 in situ hybridization. Right images, HCN4 immunofluorescence. Strong labeling is seen in the central area of the node, and no labeling in either crista terminalis, central SAN and septum interatrialis (Figure 2B through 2D).

These data indicate that the region of HCN4 expression coincides with the region defined as the central node according to standard electrophysiological and morphological crite-
ria, and that HCN4 expression, along with expression of neurofilament-M (in the rabbit) and lack of expression of atrial natriuretic peptide and connexin43, can be considered as a marker of pacemaker tissue in the adult.

Analysis of protein expression during development also confirms the link between $I_f$ and pacemaker activity. Ventricular myocytes from fetal and newborn animals contract spontaneously and express $I_f$, and disappearance of automatic rhythm and $f$-channel expression occurs with a similar time course.

$HCN4$ is a pacemaker tissue marker gene. This is implied for example by developmental data, such as those indicating that in the SAN, $HCN4$ channel expression is stimulated by Tbx3, a transcriptional repressor whose activation is a necessary step for SAN and atrioventricular bundle development and for SAN segregation from surrounding atrial tissue.

Evidence from hypertrophic and failing heart data also support an association between $HCN4$ and pacemaker activity. $I_f$ is functionally inactive in adult ventricle, both because of low expression rate of $HCN2$ (the major ventricular $HCN$ isoform in most animal species) and because too negative, unphysiological voltage range of activation. Expression of $HCN2$ (and $HCN4$), however, is strongly upregulated in cardiac hypertrophy and heart failure; as expected from the role of $I_f$ in pacemaking, this leads to increased susceptibility to ectopic rhythm.

**Pharmacological Evidence**

Pharmacological dissection is the gold-standard method to investigate the functional contribution of individual ion channels to specific aspects of cell electric activity. If funny channels have indeed a specific role in initiation of spontaneous activity and rate control, then pharmacological channel modulation should specifically affect the pacing rate. “Pure” heart rate reduction has been pursued for decades as a major pharmacological target by drug companies, because lowering heart rate, by decreasing oxygen demand and increasing diastolic time of myocardial perfusion, improves the prognostic outcome of cardiac diseases such as ischemic heart disease, angina and heart failure.

Elevated resting heart rate is also known as an independent predictor of cardiovascular morbidity and mortality and is associated with greater incidence of sudden cardiac death, a predictor of cardiovascular morbidity and mortality and is a marker of pacemaker tissue in the adult. $HCN4$ is a pacemaker tissue marker gene. This is implied for example by developmental data, such as those indicating that in the SAN, $HCN4$ channel expression is stimulated by Tbx3, a transcriptional repressor whose activation is a necessary step for SAN and atrioventricular bundle development and for SAN segregation from surrounding atrial tissue.

Evidence from hypertrophic and failing heart data also support an association between $HCN4$ and pacemaker activity. $I_f$ is functionally inactive in adult ventricle, both because of low expression rate of $HCN2$ (the major ventricular $HCN$ isoform in most animal species) and because too negative, unphysiological voltage range of activation. Expression of $HCN2$ (and $HCN4$), however, is strongly upregulated in cardiac hypertrophy and heart failure; as expected from the role of $I_f$ in pacemaking, this leads to increased susceptibility to ectopic rhythm.

The potential therapeutic applications of ivabradine might in fact extend beyond the treatment of stable angina, and...
viability for clinical use in ischemic heart disease and cardiac failure has been considered.\textsuperscript{93} The BEAUTIFUL trial (Morbidity-Mortality Evaluation of the \( I_f \) Inhibitor Ivabradine in Patients With Coronary Disease and Left-Ventricular Dysfunction) evaluated the action of ivabradine on a cohort of almost 11 000 CAD patients. The mean heart rate of patients treated with ivabradine (5 mg twice a day to 7.5 mg twice a day) slowed by 6 bpm relative to placebo; the study showed that in a subgroup of patients with heart rates \( \geq 70 \) bpm, although primary end points (cardiovascular death, admission to hospital for new-onset or worsening heart failure) were not significantly improved, secondary end points were (admission to hospital for fatal and nonfatal myocardial infarction, coronary revascularization).\textsuperscript{104} In a more recent study (ASSOCIATE), ivabradine improved exercise tolerance and delayed the development of ischemia in patients with coronary artery disease (CAD).\textsuperscript{105} In a comparable study, ivabradine (3 \( \mu \)mol/L) reduced the heart rate of patients with at least one coronary risk factor and \( >70 \) bpm by 6 bpm relative to placebo.\textsuperscript{106}

Thus, ivabradine can be used to reduce the incidence of CAD manifestations in a subgroup of high-risk patients.\textsuperscript{106} In all cases, it is important to stress that the efficacy of treatment with ivabradine is consequent to its selective funny channel block and associated reduction in heart rate slowing.

The properties of ivabradine-induced block of native funny channels\textsuperscript{101,107} and of individual HCN isoforms\textsuperscript{108} have been investigated with some detail. Low concentrations of ivabradine block funny channels with a high degree of selectivity and in a use-dependent way. Block occurs at the intracellular channel side and therefore requires drug molecules to enter the cell before acting. Use dependence manifests itself as a slowly progressing block accumulation during repetitive channel activation/deactivation cycles (Figure 3) and derives from more basic properties of ivabradine action. Specifically, the \( I_f \) block by ivabradine: (1) is an “open channel” block, i.e., channels need to be open for the drug to be able to reach the binding site within the pore; and (2) is highly voltage-dependent, being stronger at depolarized voltages.

These 2 properties are apparently in contrast, because in the first case, access to the blocking site requires hyperpolarized voltages (to open channels), and, in the second, block occurs predominantly at depolarized voltages (where channels are closed). Far from being contrasting, these properties are related: in fact, the use-dependent block occurs during repetitive open/closed channel cycling. This feature has intriguing therapeutic potential because it implies that the drug effect might be stronger at high (tachycardic) rates. It is worth noting that case reports have been published indicating successful treatment of inappropriate sinus tachycardia with ivabradine.\textsuperscript{109,110}

The voltage dependence of \( f \)-block (Figure 4) results from the positively charged nature of ivabradine, which carries a tertiary ammonium ion. Because they are positively charged, during a depolarization intracellular drug molecules will tend to move out of the cell across the channel and will therefore reach their binding site within the pore with higher probability.\textsuperscript{107} A unique property of ivabradine is that its blocking action is not voltage-dependent per se but is rather “current-dependent”; in other words, block efficiency depends on the direction of current flow. The results in Figure 4 show that the steady-state block caused by 3 \( \mu \)mol/L ivabradine during an activation/deactivation protocol (−100/−30 mV) is stronger in a low \( Na^+ \) solution, when the current at −30 mV is outward, than in normal Tyrode solution, when the current at −30 mV is inward. The “current-dependent” increase of block occurring when ions flow out of the channel is caused by a “kick-in” type of effect, whereby drug molecules are pushed from the intracellular water-filled cavity toward their binding site within the pore by the outward movement of ions. The opposite “kick off” type of effect results from the inward ionic movement during hyperpolarization, which explains why hyperpolarization relieves block.\textsuperscript{107}

Despite the tight correlation between selective \( I_f \) block and “pure” heart rate reduction, there are several limitations in the use of ivabradine (and in fact of any funny channel blocker developed so far) as a tool to quantify the exact degree of \( I_f \) contribution to pacemaking during activity. This is attributable to some specific features of ivabradine block. Firstly, selectivity for \( I_f \) block is substantial at low/moderate ivabradine concentrations, but at higher concentrations (range \( > 3 \mu \)mol/L), other channels are affected and selectivity is lost; because the \( I_f \) half-block concentration is 2.8 \( \mu \)mol/L,\textsuperscript{107} full and simultaneously selective \( I_f \) blockade cannot be achieved. Secondly, even saturating concentrations of ivabradine do not block \( I_f \) fully, as shown in Figure 4. Thirdly, block is use-dependent, and during the repetitive channel activation/deactivation cycling associated with spontaneous activity,
block will reach a steady-state level lower than maximal block; this implies that the \( I_f \) reduction caused by 3 \( \mu \text{mol/L} \) ivabradine and known to generate approximately a 30% slowing\(^44\) will certainly be, and perhaps substantially, lower than the maximal \( \approx60\% \) block shown in Figure 4. Taken together, these data indicate that ivabradine cannot be used to dissect the full \( I_f \) contribution to pacemaker activity and that at the same time this contribution is likely to be higher than the one expected on the basis of ivabradine-induced slowing.

Block of \( I_f \) by ivabradine or other blockers does not abolish autonomic rate regulation,\(^111,112\) suggesting that \( I_f \) is not necessary for rate control. However, persistence of autonomic control of rate may be partly explained if \( I_f \) is not blocked fully, because under these conditions both the \( I_f \) responsiveness to neurotransmitters and \( I_f \)-mediated autonomic rate modulation are, at least partly, preserved; as discussed above this is the case with ivabradine but also, for example, with Cs\(^+\), because Cs\(^+\) (a K\(^+\)-channel blocker) is unselective and blocks \( I_f \) only partially at diastolic voltages.\(^90\) At the same time, a contribution to autonomic rate modulation clearly also involves Ca\(^{2+}\) currents and the Na\(^+\)/Ca\(^{2+}\) exchange mechanism, which play an important role in the late fraction of diastolic depolarization.\(^113\) A detailed quantification of the exact contribution of \( I_f \) to generation and control of pacemaker polarization will be feasible when more highly specific and efficient funny channel blockers are available.

### Genetic Evidence

The search for the genetic basis of inherited arrhythmogenic diseases has advanced rapidly since the first cardiac channelopathies were described. Channelopathies are caused by defective ion channels whose normal physiological function is impaired,\(^114\) which in the heart leads to abnormalities in electric activity and/or ventricular arrhythmias,\(^115\) suggesting that \( I_f \)-mediated autonomic rate modulation is shifted 4.9 mV to more negative voltages. A and C are \( \alpha\)-helices (P is pore) and 1 to 8 are \( \beta\)-sheets according to the known CNBD sequence of hHCN4\(^117\); the S672 residue (also indicated by stick model) is close to the cAMP binding pocket. C, Mean activation curves measured in HEK293 cells expressing wild-type (wt), homozygous mutant (mut), or heterozygous wild-type\(^+\) mutant hHCN4 (wt/mut, full circles) in whole-cell (top) and macropatch inside-out conditions (bottom). Relative to wild type, the heterozygous mutation curve is shifted 4.9 mV to more negative voltages. A and C are adapted from Milanesi et al.\(^117\) Copyright © 2006 Massachusetts Medical Society. All rights reserved.

Exhaustive clinical and experimental data exist for several well-investigated cardiac channelopathies, including different types of LQTS, short QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia.\(^115\)

Despite their relevance to generation and maintenance of normal pacemaker activity, \( f \)-channels were not recognized as potential targets in the search for arrhythmia-related channelopathies until recently, perhaps because of the relatively late cloning of their molecular correlates, the HCN channels.\(^118\)

Several genetic alterations of the open reading frame of HCN4 channel gene associated with rhythm disturbances have now been described. First reports suggesting a correlation between HCN4 mutations and rhythm disturbances described a single patient with bradycardia, atrial fibrillation, and catecholaminergic polymorphic ventricular tachycardia.\(^115\)

Figure 5. Familial sinus bradycardia associated with a point mutation of HCN4 (S672R). A (top), Pedigree of a family with asymptomatic sinus bradycardia; full/open symbols refer to individuals with/without the mutation, respectively. A (bottom), Rest heart rates of family members plotted in correspondence with the pedigree plot above, as indicated by full/open symbols; all individuals with the mutation had rates below 60 bpm, whereas rates of all individuals without the mutation were higher than 60 bpm. Arrow indicates the proband. B, Three-dimensional ribbon representation of the CNBD of hHCN4, modeled on the basis of the mHCN2 CNBD crystal structure\(^116\); the bound cAMP is drawn as a stick model. A through C are \( \alpha\)-helices (P is pore) and 1 to 8 are \( \beta\)-sheets according to the known CNBD sequence of hHCN4\(^117\); the S672 residue (also indicated by stick model) is close to the cAMP binding pocket. C, Mean activation curves measured in HEK293 cells expressing wild-type (wt), homozygous mutant (mut), or heterozygous wild-type\(^+\) mutant hHCN4 (wt/mut, full circles) in whole-cell (top) and macropatch inside-out conditions (bottom). Relative to wild type, the heterozygous mutation curve is shifted 4.9 mV to more negative voltages. A and C are adapted from Milanesi et al.\(^117\) Copyright © 2006 Massachusetts Medical Society. All rights reserved.

Genetic Evidence

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and ACh concentration from the plots in C is shown for the 2 lowest ACh doses used to measure rates (10 and 100 nmol/L; empty bars); the slowing associated with the mutation, corresponding to a 4.9-mV negative shift of the activation curve, is also plotted (filled bar). This plot illustrates the fact that the mutation-associated negative shift of 4.9 mV, intermediate between shifts induced by 10 and 100 nmol/L ACh, causes a rate slowing in the family investigated that is intermediate between the shifts caused by the 2 ACh concentrations. This supports the view that the inheritable bradycardia described is fully attributable to the S672R-induced shift of the activation curve.

Although all the 3 HCN4 mutations described above occur in the C terminus, a fourth (single-point) mutation associated with familial asymptomatic bradycardia was later found in the pore region of the channel (G480R). Affected family members had an average heart rate lower than 55 bpm, whereas unaffected individuals had an average rate higher than 63 bpm. The G380R mutation appeared to reduce the amount of current available for diastolic depolarization by decreasing channel synthesis and trafficking, as well as by shifting voltage dependence of activation to a more negative range. The mutation involves the GYG selectivity sequence typical of K⁺-permeable channels, and a modified permeability might therefore have been expected. However expression of mutated HCN4 channels led to a current with the same reversal potential as wild-type channels, suggesting no significant change in the Na/K permeability ratio. Another possible alteration, the channel conductance, was not investigated.

In summary, the mutations described to date in the literature may represent specific cases of a general mechanism for sinus arrhythmias based on functional defective HCN4 channels.

Conclusions
Since the first description of If, its role in underlying generation of pacemaker activity and rate control has been investigated in detail in a variety of conditions and established on the basis of several experimental findings. Recently, practical developments of the concept of If-dependent pacemaking have shown that the properties of funny channels can be exploited in clinically relevant applications. Thus, use of “heart rate–reducing” drugs such as ivabradine, which acts by selective inhibition of the If current, allows pharmacologically controlled slowing of cardiac rate, an important tool in the therapeutic approach to ischemic heart disease and other diseases whose prognosis is ameliorated by slowing heart rate. Furthermore, certain HCN4 protein mutations are associated with inheritable cardiac arrhythmias such as sinus bradycardia, suggesting the existence of a general mechanism for rhythm disorders based on altered function of funny channels. Finally, exporting funny channels to silent cardiac tissue through either gene- or cell-based protocols represents a viable tool for the future development of biological pacemakers eventually able to replace electronic ones. Further knowledge of the molecular details of funny channel structure and function will likely allow in the future a more efficient and clinically relevant approach to cardiac rate control.
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