Dario DiFrancesco

Synopsis of work

The heart of an adult person beats normally some 100,000 times a day, with a fairly regular rhythm. The heart rate is finely regulated at any time during our life according to need: for example, it slows at night and accelerates at wake-up, and changes rapidly during physical exercise or in response to varying metabolic and emotional conditions. A major physiological controller of heart rate is the autonomic nervous system, which causes acceleration by sympathetic stimulation and slowing by vagal stimulation through the release of the neurotransmitters noradrenaline and acetylcholine, respectively. How is the cardiac spontaneous activity generated, and how is rate controlled so rapidly and efficiently? These are central questions which have always attracted the interest of physiologists and cardiologists.

Just over a hundred years ago, Keith and Flack first observed that the heartbeat originates from a special region within the right atrium, the sinoatrial node (SAN). Only about seventy years later, however, did the cellular basis for the initiation of the heartbeat become apparent thanks to microelectrode recordings of electrical activity. Indeed, cells from the SAN region, termed "pacemaker" cells, generate action potentials with a special shape, characterized by the presence of a phase called "pacemaker" or "slow diastolic" depolarization. After termination of an action potential, rather than stabilizing to a certain "resting" level as in working atrial or ventricular myocytes, the membrane potential of a pacemaker cell slowly depolarizes until reaching threshold for another action potential, thus giving rise to spontaneous activity.

The discovery of the cellular mechanism underlying diastolic depolarization and driving pacemaker activity and control of heart rate has been the major achievement of the early research of Dario DiFrancesco.

In the late '70s, working in Denis Noble's laboratory in Orford with Hilary Brown and Susan Noble, the author described for the first time the cardiac so called "pacemaker" current, a large inward current which slowly activated on hyperpolarization within the range of diastolic voltages. The current had very atypical biophysical properties, since no other known current at the time had similar features, and was dubbed "funny" (I_f) for this reason. These features were, however, perfectly suited for generating the diastolic depolarization phase of the action potential of pacemaker cells, hence spontaneous activity. So, I_f could be a real "pacemaker" current! A second finding made during the same study was even more striking: I_f was increased by adrenaline, and could therefore be the current responsible for the acceleration of rate during sympathetic stimulation.

These were breakthrough findings which identified what appeared to be the long sought cellular mechanism able to induce spontaneous activity in pacemaker cells. According to this mechanism, pacemaking was generated by activation of the funny current at the end of an action potential, which initiated diastolic depolarization; also, the funny current controlled the rate of diastolic depolarization, hence the heart rate, and was responsible for frequency acceleration during adrenergic stimulation.

These first basic findings were published in 1979 in a Nature paper, and were followed by several studies in the following years. Many more data were collected by the author and by other laboratories on the properties of the funny current which further confirmed its role in the generation of cardiac pacemaking and in the control of rate. Some of the most relevant findings are outlined below.

In 1981, a major advance in the understanding of pacemaking was the reinterpretation of the pacemaker mechanism in another type of myocyte capable of spontaneous activity, the Purkinje fibres of conduction tissue. The pacemaker mechanism thought at the time to operate in these cells was the decay of a potassium current, the so called I_{K2} current, occurring during diastole. This theory was well established and universally accepted by the scientific community. In two companion papers in the Journal of Physiology, Dario DiFrancesco showed that I_{K2}, previously interpreted as a K+ current, was

instead a disguised funny current, and that the two pacemaker mechanisms in SAN cells and Purkinje myocytes were therefore not different but just the same, mediated in both cases by activation of the inward funny current during diastole. This result allowed for the first time an integrated view of pacemaking in the heart. A theoretical model incorporating these and other experimental data was developed in collaboration with Denis Noble. This model, the first ever to investigate numerically the ability of the funny current to underlie the diastolic depolarization and the generation of rhythmic activity, indeed allowed the reconstruction of spontaneous action potentials and represented the paradigm from which subsequent cellular models of the heart were developed.

In 2015, the Royal Society of London celebrated the 350th years of the **Philosophical Transactions**, the first and oldest scientific journal ever. The celebration included several activities (see: https://royalsociety.org/journals/publishing-activities/publishing350/history-philosophical-transactions/)
among which the collection and review of the most influential papers published in its long life. Starting from some 600 of its best performing papers, a selection committee filtered through and eventually identified 16 papers in Phil Trans A (physical sciences) and 17 papers in Phil Trans B (life sciences) as the best papers in 350 years. Authors included Isaac Newton, Michael Faraday, James Joule, James Clerk Maxwell, Osborne Reynolds, Antoni van Leewenhoeck, Hans Sloane, Edward Stone, Alan Turing, Peter Medawar, David Marr to name a few.
Denis Noble and Dario DiFrancesco were ecstatic to realize that one of the 17 papers selected in Phil Trans B was the model paper written in 1985: DiFrancesco, D. & Noble, D. (1985) A model of cardiac electrical activity incorporating ionic pumps and concentration changes. Phil. Trans. R. Soc. Lond. B 307, 353-398; see
http://rstb.royalsocietypublishing.org/content/370/1666)
https://doi.org/10.1098/rstb.2014.0316
https://royalsociety.org/blog/2015/04/a-conversation-with-dario-difrancesco-and-denis-noble/

Denis Noble was the only living author attending a special Royal Society ceremony in March 2015, and released this comment:
"It felt deeply moving to be present at this magnificent celebration as, I believe, the only representative of such a pantheon of greats in science from Isaac Newton and Antonie van Leeuwenhoek in the seventeenth century to Alan Turing and Peter Medawar in the twentieth. It is both an honour and a deeply humbling experience. No living scientist naturally feels it right to be included. I said nothing but felt their presence 'standing on the shoulders of giants', to quote Newton himself."

The first single-channel recording of I_f was reported by the author in 1986 in a Nature paper. This was a particularly demanding achievement because of the very small single-channel conductance (about 1 pS), today still among the smallest single-channel recordings ever published. This difficulty is reflected by the fact that only 20 years later have similar recordings been made by another laboratory, which fully confirm the 1986 results.

Another important result obtained in the mid '80s concerns the vagal modulation of heart rate. Until the late ‘80s, the generally accepted interpretation of the action of the vagus nerve on rate was that parasympathetic-induced slowing is due to activation of an acetylcholine-dependent K+ current in pacemaker cells. However, the existence of a pacemaker mechanism based on funny channels raised the question whether this latter mechanism could also participate in the vagal control of rate. In a series of papers in 1987-1989 from the author's lab, it was found that acetylcholine had a strong inhibitory action on the funny current, according to a mechanism opposite to that exerted by noradrenaline. These findings challenged the established view of a K+ permeability increase as the cellular process underlying vagal control of rate. Indeed, the author and collaborators showed in a 1989 paper published in Science that the funny current inhibition, and not the K+-conductance activation, was responsible for the slowing of pacemaker rhythm at low acetylcholine concentrations.

In the early '90s, in a publication in Nature, DiFrancesco demonstrated for the first time the action of the intracellular second-messenger cAMP on funny channels. This molecule acted by direct binding to the channel protein, and not by a phosphorylation process. Activation by cAMP represented the mechanism responsible for I_f increase by sympathetic stimulation, and for I_f inhibition by parasympathetic stimulation. An important consequence of these findings is that they led to an integrated view of the entire process by which the autonomic nervous system modulates heart rate by means of the funny current. In brief, sympathetic stimulation leads to increased intracellular levels of cAMP, a consequent increase of the funny current, and therefore to an increased slope of diastolic depolarization and rate acceleration; conversely, parasympathetic stimulation leads to exactly the opposite set of events and therefore to rate slowing.

These fundamental early discoveries, integrated by other data such as the cloning of the molecular correlates of native funny channels (the HCN channels, for Hyperpolarization-activated, Cyclic Nucleotide-gated), have eventually led to the development of major clinically-relevant applications.

1. Application to the genetics of cardiac rhythm disorders. Of the 4 HCN channel isoforms known, HCN4 is the most highly expressed in the sinoatrial node (SAN). In one of the first studies investigating the potential link between dysfunctional HCN4 channels and Sinus Node Disease, the author's lab showed that a form of asymptomatic sinus bradycardia, found in a large Italian family, is associated with a point mutation of HCN4. The mutation is loss-of-function, i.e. it reduces I_f during diastole, thus slowing spontaneous frequency and generating bradycardia. This result is important in that it represents a specific case of a broader mechanism for rhythm disturbances based on constitutive alterations of funny channels.

A few years later, also a gain-of-function mutation was found and reported by the PaceLab. In perfect agreement with the functional role of the funny current according to which increasing the I_f availability is bound to accelerate the heart, this mutation led to a tachyarrhythmia (Inappropriate Sinus Tachycardia, IST).

A large number of cases where rhythm disorders are caused by HCN4 mutations have been reported by several laboratories including that of the author, and more are likely to be found with further investigation.

2. Application to the field of "biological pacemakers". These are auto-rhythmic cellular substrates able to induce or control pacemaker activity, which may eventually replace the electronic pacemakers used today. These new biological devices are based upon the induction of pacemaker function in silent cardiac tissue by the transfer of funny (or HCN) channels. This can be achieved by means of viral transfection or by transfer of cells engineered to overexpress HCN channels, or expressing funny channels constitutively. The PaceLab has shown that the isolation from mouse Embryonic Stem cells of a non-teratogenic population of cardiac precursors is able to mature and form a fully functional pacemaker-like tissue.

While several proof-of-principle experiments have shown that biological pacemaking is indeed possible, the road to its clinical application is still a long one.

3. Application to pharmacological control of heart rate. This is the most important application of the concept of funny channel-based pacemaking, since it has led to the development of therapeutic tools now commercially available for the cure of specific cardiac diseases.

The beneficial effect of heart rate slowing in cardiac diseases such as angina, ischaemic heart disease and heart failure is well known; also known is the association between high resting heart rate and cardiovascular or all-cause mortality. Presently used therapies with β-antagonists and calcium blockers ameliorate mortality risk in part because of their heart rate-slowing effects. However, these drugs affect other cardiovascular and non-cardiovascular parameters and may also have adverse side-effects (for example a reduced inotropic function).

Since funny channels have a basic role in heart rate modulation, they clearly represent an important target for the development of drugs aimed to specifically control heart rate, without

complicating side effects. Several drugs acting on funny channels have indeed been developed by drug companies in the last few decades, with the aim of reducing heart rate specifically. Among them ivabradine, developed by Servier, is the only drug having passed all clinical tests. The PaceLab has clarified some molecular aspects of the mechanism of action of ivabradine on funny-channels and consequent heart rate-reducing effect. These results provide a detailed explanation for the "use-dependence" of ivabradine, a clinically very useful property according to which high rates are affected more vigorously than slow rates.

Ivabradine is available with the commercial name of Procoralan or Corlentor (also other brand names), and is prescribed against chronic *angina pectoris* and heart failure. Many clinical trials, some quite large, have investigated the clinical efficiency of ivabradine in reducing morbidity/mortality of coronary heart disease and systolic heart failure patients, among which BEAUT*I*fUL, SH*I*fT, SIGN*I*fY, MOD*I*fY, ED*I*fY, and others. In fact, 22 trials on ivabradine are listed in the Servier Trials web page (https://clinicaltrials.servier.com/?s=ivabradine).

In summary, the discovery of the funny current and its role in the generation of spontaneous activity and control of heart rate has been a fundamental achievement in the understanding of the basic physiology of the heart, and has also resulted in the development of important clinical applications.

Dario DiFrancesco

Summary of scientific activity

(Numbers of quotations refer to the list of publications)

A major contribution given by the author to the field of cardiac physiology is the discovery of the cardiac pacemaker ("funny") current in the sinoatrial node, the pacemaker region of the heart, and of its involvement in the mechanism underlying generation of pacemaker activity and control of heart rate by the autonomic nervous system.

As a postdoc in Cambridge in 1976, and then in Oxford in 1977-80, the author joined the group led by Denis Noble on several projects on the properties of different ionic currents in Purkinje fibres and in amphibian atrial and sinus venosus tissues (1, 3, 7, 8). During this period, he visited the laboratory of Wolfgang Trautwein in Homburg/Saar, where together with Akinori Noma applied the voltage clamp technique to small mammalian sinoatrial node (SAN) preparations to study the properties of the ACh-activated current (5, 6). He brought back to Oxford the technique for voltage clamping SAN pacemaker tissue, and in collaboration with Hilary Brown and Susan Noble, he performed the first experiments demonstrating the existence of an ionic current, termed I_f (f for "funny"), with unusual properties: I_f was activated on *hyperpolarization* and *inward* in the diastolic range of voltages, properties perfectly coherent with the idea that activation of this current was involved in the *generation* of the diastolic depolarization phase of the action potential, i.e. was responsible for *spontaneous activity*.
Confirming the importance in pacemaking was the evidence that, as well as generating the slow diastolic depolarization process, I_f had a major role also in determining the cardiac *rate acceleration* induced by *adrenaline* (2, 4) (Figure 1).

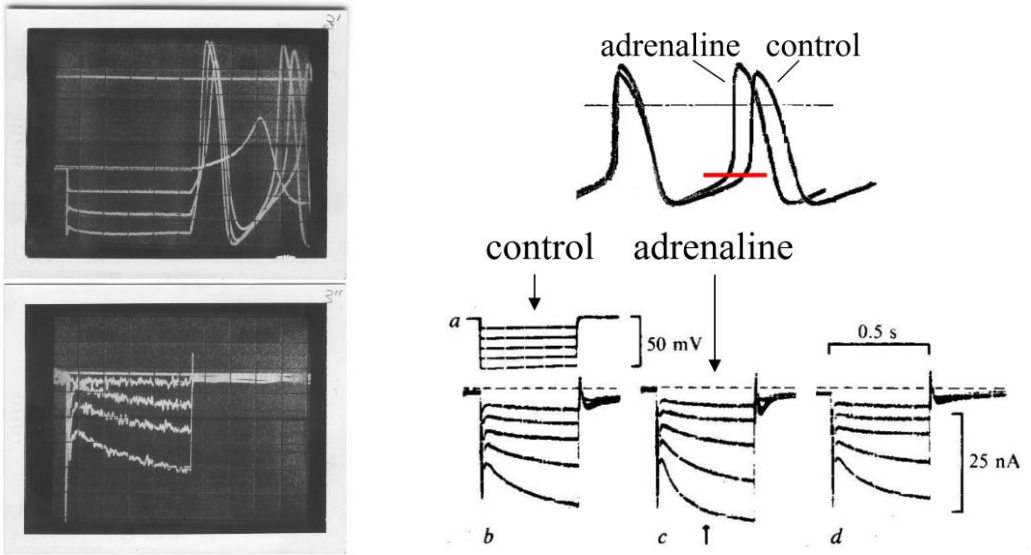


Fig. 1. Left, original polaroid photographs of Tektronix memory screen showing that I_f is activated within the diastolic depolarization voltage range. Oxford, Laboratory of Physiology, autumn 1978. Right, upper, rate acceleration in adrenaline is associated with increased steepness of diastolic depolarization. Lower, adrenaline increases I_f. Modified from Brown et at, 1979 (ref. 2).

The "funny" current introduced a new concept able to explain generation of rhythmic activity

and heart rate control. It had a great impact in cardiac physiology, but problems remained. For example, how was it possible to have two pacemaker currents? Spontaneous pacemaking in Purkinje fibres was indeed thought, at the time, to be generated by the *decay of a K^+ current*, the I_{K2} current (Noble & Tsien 1968), rather than the *activation of an inward current*, like I_f in SAN cells. This implied the paradoxical, counterintuitive consideration that pacemaking in Purkinje fibres (K^+ current decay) and in the SAN (I_f activation) were different processes.

This puzzle was solved a couple of years after the first description of I_f, with the demonstration that the I_{K2} current simply did not exist: it was no less than a camouflaged I_f! A first major breakthrough on pacemaker mechanisms fuelled by the I_f discovery in the SAN was indeed the **re-interpretation of the I_{K2} current** in Purkinje fibres and the consequent re-interpretation of the established theory of pacemaking, considered like a dogma by the scientific community (the gK-decay hypothesis: Draper & Weidmann 1951; Trautwein & Kassenaum, 1961; Noble 1962; Vassalle 1966). This theory "turned upside down", as later stated by Denis Noble himself in his renowned Physiological Society Review Lecture of 1984 (Noble, 1984).

In a set of experiments where the Purkinje I_{K2} was compared with the nodal I_f, the author showed that K^+-depletion could distort the time-course of I_{K2} during voltage clamp, and that the Purkinje fibre I_{K2} was, in fact, a mixed Na^+ and K^+ current activated on hyperpolarization like the nodal I_f (11, 12).

Different experimental evidence showed this: 1) the cell membrane conductance increased, rather than decrease, during current development on hyperpolarization (Fig. 2A); 2) the current was blocked by Cs^+ but curiously, although Cs^+ block was known, nobody had realized that the block was that of an *inward*, not of an *outward* current (Fig.2B); 3) the decay of I_{K2} during hyperpolarizing steps could be "transformed" into activation of I_f, in a Purkinje fibre perfused with Ba^{2+}, a maneuver able to block an overlapping, unwanted inward-decreasing component due to extracellular K^+-depletion occurring when large hyperpolarizing steps were applied to the preparation. (Fig. 2C).

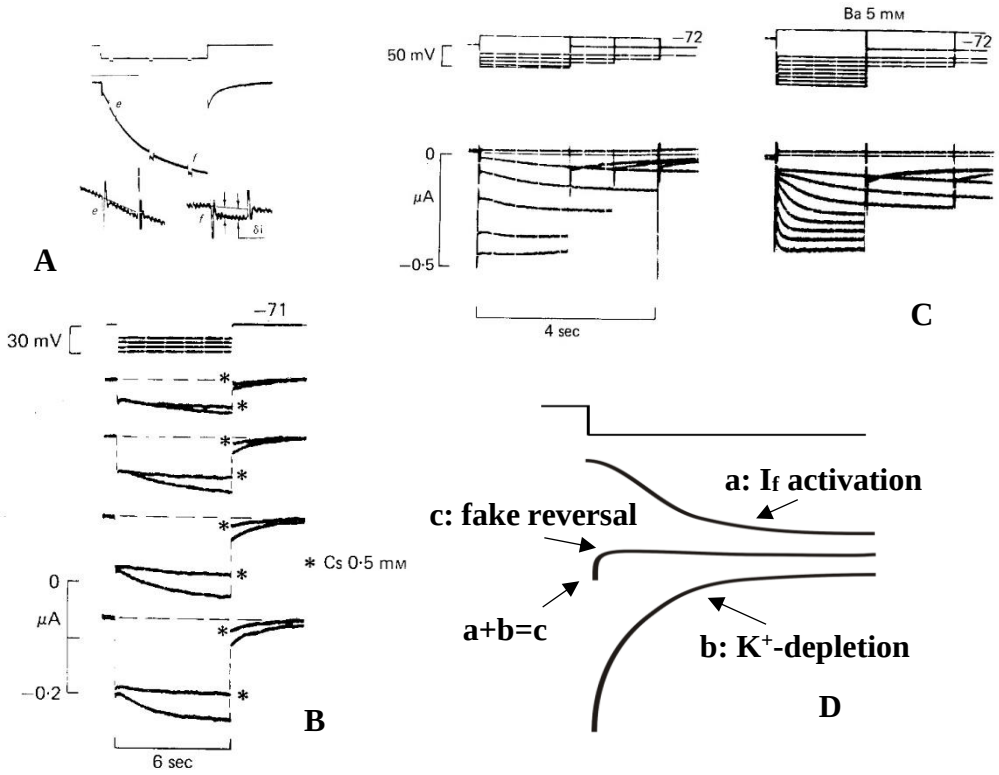


Fig. 2. Explanation in text. Modified from DiFrancesco, 1981a (ref. 11).

The tricky issue was that the apparent reversal potential (trace c in Fig. 2D), and more in general

the apparent pure K^+-current behaviour, was the result of two distinct, overlapping events: the activating I_f (trace a) and an inward-decaying K^+ current (trace b) due to K^+ depletion in extracellular clefts. So, for over a decade the description of the current I_{K2}, that "was regarded as perhaps the most fully analysed current in the heart and served as a model for other systems" (Noble, 1984), and the resulting pacemaking theory anchored to this concept, were based on misleading, technically flawed experimental data.

As stated by Noble in his 1984 review, with the new I_f model "some important and awkward details of the experimental results then receive very natural explanations". These awkward details included the disappearance of the current in zero Na^+ external solutions, a result difficult to explain for a pure K^+ current, and the reversal potential more negative than the Nernst equilibrium voltage for K^+ ions (Noble & Tsien 1968).

Thus, the concept of g_K-decay hypothesis had remained undisputed and had baffled the scientific community for 3 decades, since the original interpretation of the results of Silvio Weidmann's seminal experiments (Weidmann 1951). As well as resolving previous "awkward details", the finding that the pacemaker process in different types of spontaneously beating cells was the same strongly reinforced the concept of I_f -based pacemaking.

The late Professor Edouard Corabouef of the University of Paris-Orsay, whom the author visited for several short periods in the early 80's, described the I_{K2} re-interpretation "a little revolution" in the cardiac field. The inset below is a photographic copy of a part of the report written by E. Coraboeuf in 1981 ("Avis sur la candidature de M. DiFrancesco a un post de chercheur", 26 janvier 1981) concerning the author's results:

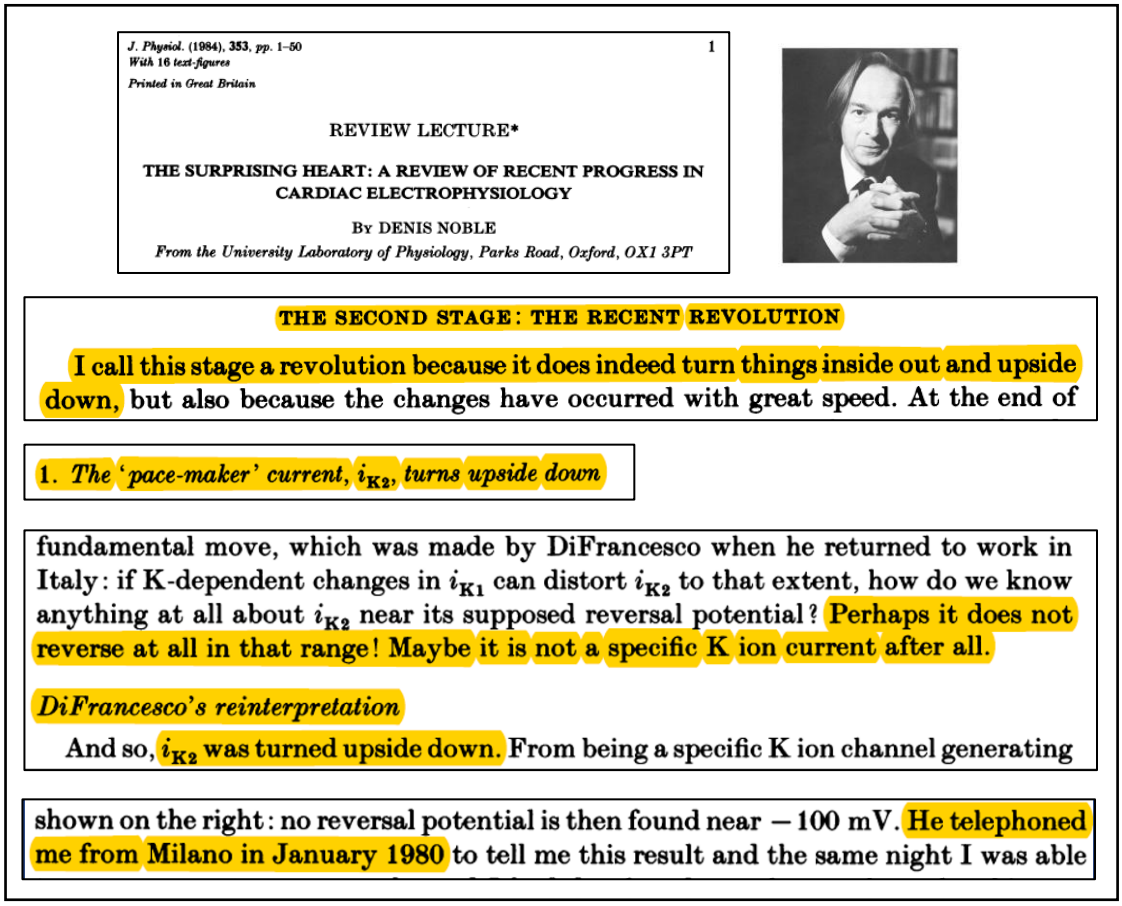
AVIS SUR LA CANDIDATURE DE Mr D. DIFRANCESCO A UN POSTE DE CHERCHEUR

Il est, en particulier, en ce qui concerne l'analyse des mécanismes responsables de l'automatisme du tissu conducteur cardiaque (fibres de Purkinje), l'auteur d'une véritable petite révolution, comme on en enregistre dans la plupart des domaines tous les 20 ou 30 ans. Le fait qu'il vienne, en effet, de renverser un dogme établi et indiscuté depuis 1955, fait qu'aujourd'hui son nom est connu des électrophysiologistes cellulaires du monde entier.

A "recent revolution" was also the way Denis Noble referred to the I_{K2} reinterpretation in his renowned Annual Review Lecture of 1983, where he described the telephone call received from Milano in 1980 and the events leading to the realization that "i_{K2} was turned upside down" (Noble, 1984, Fig. 3). This is more amply discussed below. In one of his last reviews, also the late Edward Carmeliet, one of the Fathers (with Silvio Weidmann and Eduard Coraboeuf) of Cardiac Electrophysiology, referring to the events leading to the I_{K2} reinterpretation, uses the words: "Start of The (R)evolution: Fake Reversal Potentials and Better Preparations" (Carmeliet, 2019).

In the early 80's the properties of I_f in SAN cells and Purkinje fibres were then described with greater detail in terms of ionic, kinetic and pharmacological properties (13, 14, 15). Among the early findings an important one was the mixed Na^+-K^+ ionic nature of the current, another unfamiliar property of I_f. The mixed ionic permeability explains the reversal potential of the current around -10 mV, which means that the I_f current is inward (and activating on hyperpolarization) in the pacemaker range of voltages, the perfect properties for a mechanism contributing to the generation of a depolarizing process.

A second breakthrough triggered by the I_f discovery derived from the need to model numerically the electrical activity of pacing cells, and more specifically of Purkinje fibres. Existing models using the (incorrect) I_{K2} description had been shown to reproduce faithfully several aspects of Purkinje fibre's activity, including spontaneous activity and response to adrenaline (McAllister, Noble & Tsien, 1975). How was it possible? How could an "upside down" description of the pacemaker current explain the recorded spontaneous activity? The Purkinje fibre's model obviously needed to confront with the new I_f-based theory.



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With 16 text-figures
Printed in Great Britain

REVIEW LECTURE*

THE SURPRISING HEART: A REVIEW OF RECENT PROGRESS IN CARDIAC ELECTROPHYSIOLOGY

By DENIS NOBLE

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THE SECOND STAGE: THE RECENT REVOLUTION

I call this stage a revolution because it does indeed turn things inside out and upside down, but also because the changes have occurred with great speed. At the end of

1. *The 'pace-maker' current, i_{K2}, turns upside down*

fundamental move, which was made by DiFrancesco when he returned to work in Italy: if K-dependent changes in i_{K1} can distort i_{K2} to that extent, how do we know anything at all about i_{K2} near its supposed reversal potential? Perhaps it does not reverse at all in that range! Maybe it is not a specific K ion current after all.

DiFrancesco's reinterpretation

And so, i_{K2} was turned upside down. From being a specific K ion channel generating

shown on the right: no reversal potential is then found near −100 mV. He telephoned me from Milano in January 1980 to tell me this result and the same night I was able

Fig. 3. Explanation in text. Excerpts from Noble, 1984.

When the author, working in Milano upon return from Oxford, had collected enough convincing experimental evidence to prove that the I_{K2} current simply did not exist, and was an I_f current "turned upside-down", he decided to communicate this to Denis Noble. As mentioned above, this was done in 1980 by telephone, an atypical way to communicate science. This telephone call has been recalled by Denis Noble himself in some of his most notorious reviews (see Fig. 3; Noble 1984; Noble 2021). This communication was the basis for a valuable, continuous collaborative work that led to development of a novel theoretical model incorporating the new I_f interpretation and other new data on Na^+, Ca^{++} and K^+ currents, Na-K pump, Na-Ca exchange and the dynamics of intracellular and extracellular ion concentrations (17). The model allowed to interpret all experimental data, and represented the paradigm from which subsequent cellular models of the heart were developed (see Note 1).

Note 1. This model will later receive significant public recognition by the scientific and academic communities. In 2015, the Royal Society of London celebrated the 350th years of the Philosophical Transactions (20) by selecting the most influential papers published since 1665. The selection process eventually identified 33 articles, including papers by Isaac Newton, Michael Faraday, James Joule, James Clerk Maxwell, Osborne Reynolds, Antoni van Leewenhoeck, Hans Sloane, Edward Stone, Alan Turing, Peter Medawar, David Marr to name a few. Among these giants, also selected was the DiFrancesco & Noble model paper written in 1985 (Dibb et al 2015) (21-22).

Further progress in the understanding of the I_f properties came in 1986 with the recording of single f-channels (20). Several laboratories had failed to record single f-channel activity. This was achieved only thanks to a modification of the patch-clamp technique, performed by using two pipettes on the same cell. This protocol was particularly demanding, but at the same time provided a much greater resolution of single-channel activity than could be obtained by the traditional single-pipette technique (Fig. 4 left). Such a modification was necessary due to the extremely small I_f single-channel conductance (about 1 pS, Fig. 4 middle).

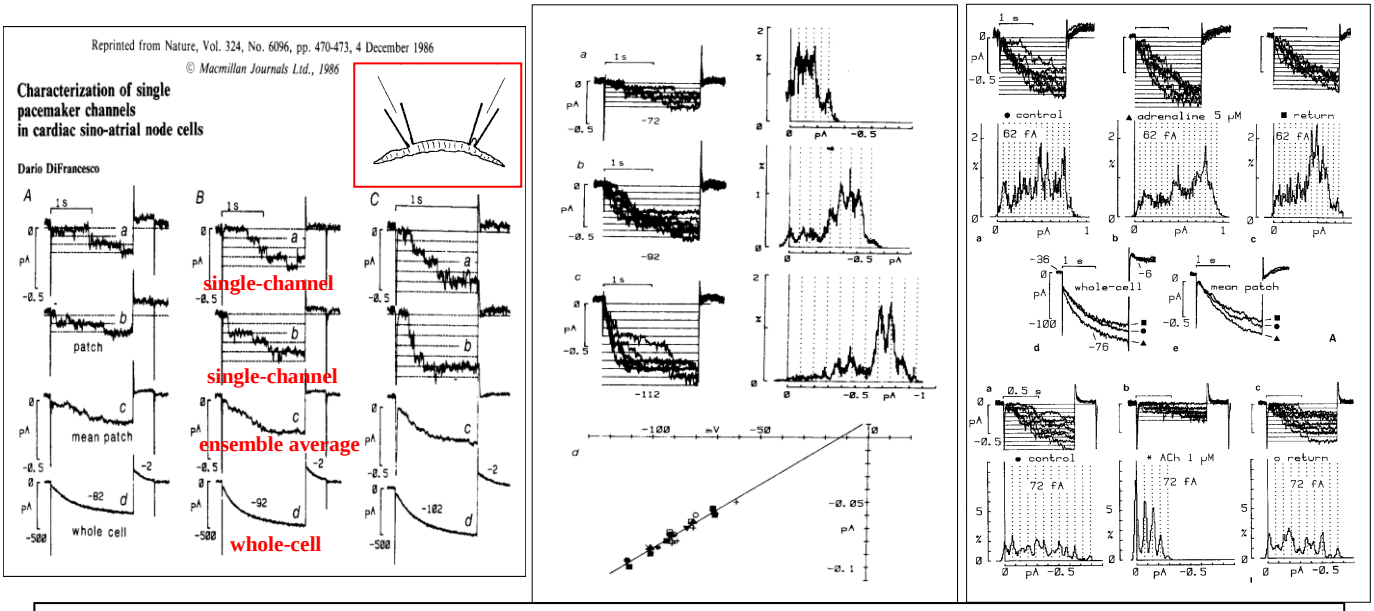

Fig. 4. Single f-channel recording. Left, sample recordings with two pipettes, one in whole-cell (voltage steps application) and the other in cell-attached (single channel recording) configuration. Middle, single channel I/V curve yielding a single-channel conductance of 0.98 pS. Right, adrenaline (top) and ACh (bottom) increase/decrease the probability of channel opening, respectively, without modifying channel conductance. Modified from DiFrancesco (1986), DiFrancesco & Mangoni (1994) (refs. 20, 39).

The 1986 experiments, and others performed on I_f by the author in a later investigation made in collaboration with Matteo Mangoni with the inside-out patch recording protocol (39), represent among the smallest direct single-channel recordings made with the patch-clamp, a view shared by Erwin Neher, the Noble-Prize winner who developed the single-channel measuring technique.

The difficulty of recording the activity of such a small single channel is confirmed by the fact that 20 years went by before similar recordings of single channel currents were made in another laboratory (Dekker & Yellen, 2006) on the HCN2 isoform of HCN channels (the molecular correlates of native funny channels, see later). The very low conductance of single funny/HCN channels has then been confirmed by more single channel recordings (Thon et al 2013) and other indirect means (Johnson and Zagotta, 2005; Kole et al., 2006)

I_f single-channel measurements also allowed to establish the mode of action of adrenaline and ACh on I_f: it was shown that adrenaline activates, and ACh inhibits, I_f by increasing/decreasing, respectively, the probability of channel opening upon hyperpolarization, without changing the single f-channel conductance (20) (Fig. 4 right).

A more complete understanding of the functional properties of the pacemaker current in the SAN was obtained by studies of the I_f neuromodulation. In 1987, the author and collaborators demonstrated the inhibitory action of acetylcholine on I_f (21, 22, 23). The inhibition by ACh involved a shift of the current activation curve to more *negative* voltages, an effect opposite to the current stimulation exerted by Adrenaline via a *positive* shift (Fig. 5). The opposite effects of sympathetic *vs* vagal transmitters provided clear evidence for the most fundamental physiological function of the current in the heart, i.e., that of mediating the autonomic control of heart rate (139, 172).

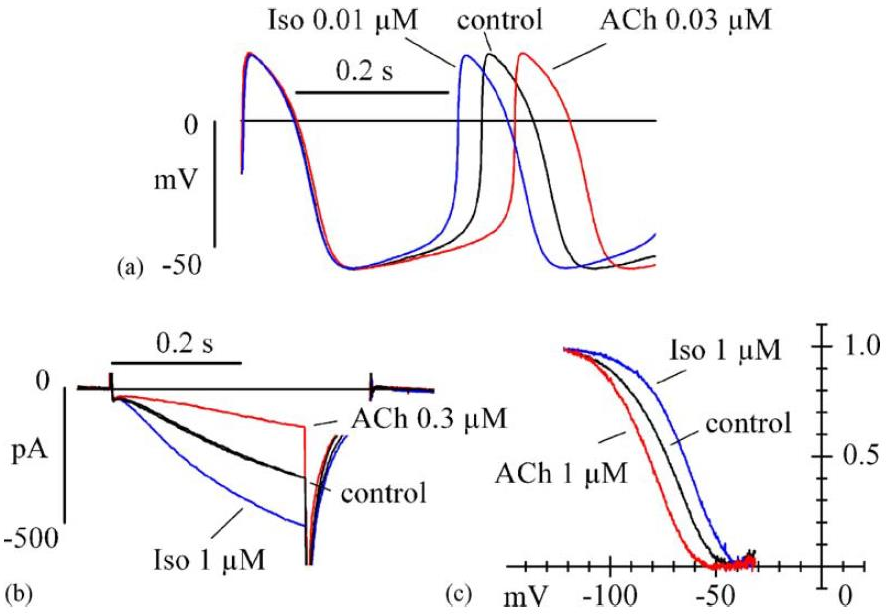

Fig. 5. The funny current mediates heart rate modulation by the autonomic nervous system. (a) Isoprenaline accelerates and ACh slows spontaneous rate in an isolated SAN cell. (b) Isoprenaline increases and ACh decreases I_f in voltage-clamp recordings, causing action potential rate changes compatible with the ones observed in (a); (c) the changes are caused by a rightward and a leftward shift of the activation curve, respectively. From DiFrancesco, 2006 (ref. 154).

As well as representing a physiological process of basic importance to the autonomic control of heart rate, the finding of the inhibitory action of ACh on I_f was also relevant because it modified the generally accepted view that cardiac rate slowing due to vagal stimulation occurs through activation of a K^+ permeability (the ACh-activated K^+ current, I_{KACh}). In a study where the activation of K^+ permeability by ACh was compared with the ACh-induced I_f inhibition, the author and collaborators showed that ACh inhibits I_f at 20-fold lower concentrations than those required to activate the current I_{KACh} (Fig. 6). According to these data, the I_f inhibition, and not the K^+-conductance activation, is the process responsible for the slowing of pacemaker rhythm at low ACh concentrations and the maintenance of "vagal tone" at rest (25).

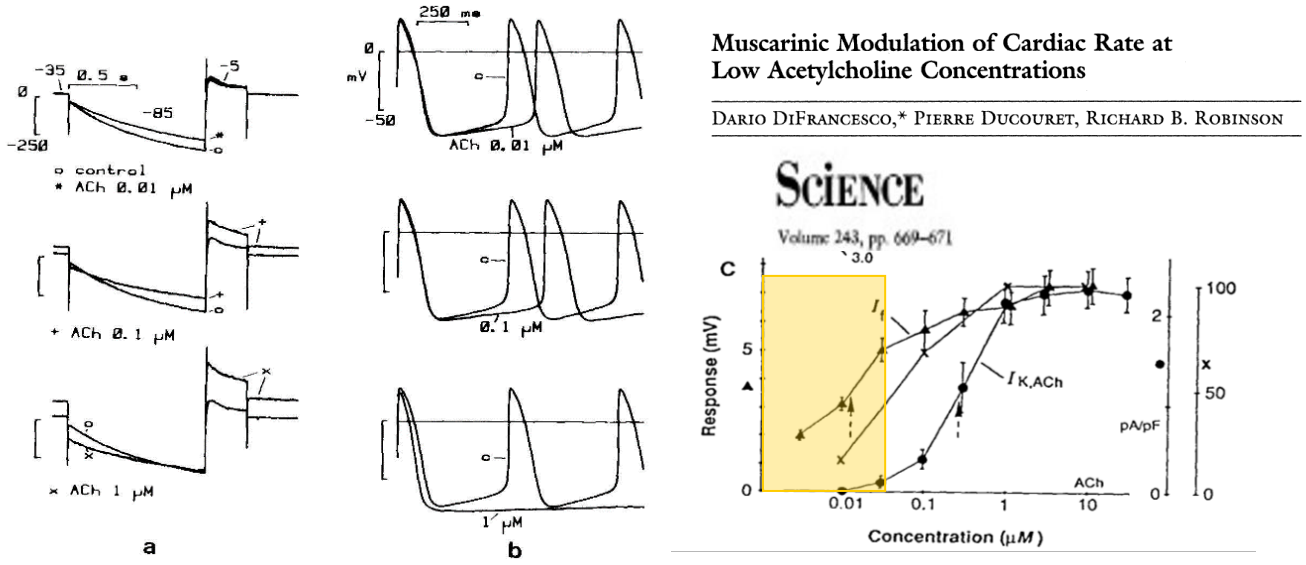

Fig. 6. Low ACh doses inhibit I_f but do not activate I_{KACh} (left) and slow rate substantially (middle). Right, dose-response relations show that I_f inhibition requires 20-fold lower ACh doses than I_{KACh} activation. From DiFrancesco et al, 1989 (ref. 25).

Further studies of the f-channel neurotransmitter-induced modulation then revealed that cAMP, the second-messenger controlling I_f, activates f-channels by direct binding to the channel protein, in the absence of any cAMP-dependent phosphorylation process (31). Thus, f-channels behave quite differently from other channels (such as Ca2+ and delayed K+ channels) which are controlled by cAMP via phosphorylation. These studies were performed using inside-out macro-patch preparations, which allow test intracellular solutions to be perfused directly on the internal side of the channel (Fig. 7).

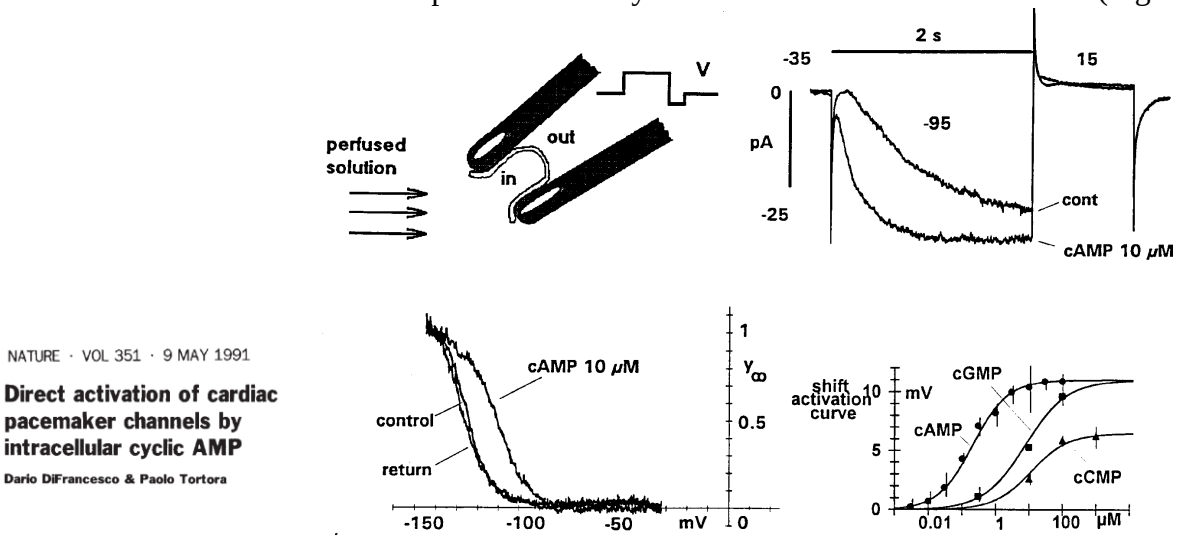

Fig. 7. cAMP directly activates funny channels. Top left, measurements made in inside-out macro patches; right, cAMP activates the I_f in a macro patch. Bottom left, cAMP action is to shift the open probability curve to more positive voltages; right, dose-response relations of mean activation curve shift induced by different nucleotides. Data redrawn from DiFrancesco & Tortora, (1991) and from DiFrancesco (1995) (refs 31, 141).

Single-channel measurements were also performed showing that, in agreement with the action of adrenaline previously reported, cAMP activates f-channels by increasing the probability of channel opening, and does not affect the single-channel conductance (39). cAMP-induced activation was the first evidence that funny channels had properties similar to what at the time was an apparently very distant family of channels, the cyclic nucleotide-gated (CNG) channels mediating sensory transduction in the retina, olfactory neurons etcetera. Indeed, several years later, in the late '90s, cloning of the HCN channels (the molecular components of funny channels) demonstrated that funny and CNG channels do belong to the same superfamily, confirming the original observation of direct f-channel activation by cAMP (31).

The neuronal funny current (current I_h)

In parallel with studies aimed to a better understanding of the properties of I_f and its role in underlying generation and control of cardiac pacemaker activity, the author contributed to studies of the properties of a similar hyperpolarization-activated current (I_h -current) in neuronal cells. In the 80's and 90's, it had indeed become clear that funny channels have important roles in a variety of cellular mechanisms in different types on neurons. These include the control of excitability, the involvement in sensory perception and transduction, the modulation of rhythmic firing and the involvement in the control of synaptic strength. Some of these aspects were investigated by the author (38, 41, 53, 55, 58).

HCN channels

The molecular correlates of native funny channels constitute the HCN family of channels (hyperpolarization-activated, cyclic-nucleotide gated channels) of which 4 mammalian isoforms have been isolated and cloned in the late 90's. The author's lab has been involved in the cloning of isoforms of the HCN family of channels (hyperpolarization-activated, cyclic-nucleotide gated channels) and to the characterization of the properties of HCN clones and native f- channels, providing relevant contributions to the knowledge of the molecular basis for channel activity (66, 68, 70, 73, 75, 83, 84, 92, 99, 102, 104, 108, 112, 113, 118, 120, 121, 129, 132). In particular it was shown that the HCN4 isoform is, among the 4 HCN isoforms known, the most relevant in the pacemaker region of the sinoatrial node (77).

Other contributions have concerned different channel types (45, 46, 52, 63, 65, 67, 69). Among these, relevant was the cloning and characterization of one of the smallest known ion channels, the first K^+ channel found in the genome of a virus (61).

Numerical modelling

Beyond the prized numerical model paper with Denis Noble to reconstruct the activity of Purkinje fibres (17), the author has contributed to numerical modelling studies of spontaneous activity in SAN cells, in collaboration with Stefano Severi (106, 116).

Exploiting funny channel properties for clinical use

While born as a basic concept of cardiac physiology, the funny channel-based pacemaker mechanism has become, in the course of decades of investigation of its properties, also an important tool in clinically-relevant applications, which highlight the functional role of I_f in pacemaking, but also provide means to exploit its properties for future development of therapeutic interventions.

There are three important applications: 1. HCN-based biological pacemaker; 2. Genetics of HCN-linked arrhythmias; 3. HCN4 pharmacology and heart rate control.

1) HCN-based biological pacemakers

Several laboratories have used different techniques in the attempt to develop "biological pacemakers", i.e. autorhythmic cellular substrates able to induce or control pacemaker activity. The

ultimate goal is to replace the electronic pacemaker used today with biological ones. Some of the protocols adopted rely upon the induction of pacemaker activity by overexpression of HCN channels via viral transfection or by transfer of cells engineered to overexpress HCN channels (such as mesenchymal stem cells) and fusion between HCN-expressing fibroblasts and myocytes.
Other approaches utilize cells which are basically autorhythmic, such as Embryonic Stem (ES) cells differentiated towards myocytes expressing funny channels and able to pace spontaneously, or HCN-expressing induced Pluripotent Stem Cell (iPSC)-derived cardiomyocytes. The results collected so far show that HCN transfer can indeed be a viable method to generate biological pacemakers, although the evidence is still mainly only proof-of-concept. This field is continuously expanding and has been reviewed thoroughly (Robinson et al., 2006; Rosen et al., 2011; Chauveau et al., 2014).

2) Genetic application

The hypothesis that I_f plays a role in generation of spontaneous activity and control of heart rate implies that functional defects of funny channels can affect the generation and maintenance of normal rhythm. In this case, one could expect to find rhythm disturbances which are attributable to mutated funny channels. The author's lab has screened several cardiac patients with rhythm disturbances such as bradycardia, tachycardia, Atrial Fibrillation, Atrio-Ventricular block and other types of arrhythmias. Early work has shown the presence of a causative point mutation of HCN4 in a large Italian family with asymptomatic sinus bradycardia (86). The S672R is a loss-of-function mutation localized in the Cyclic Nucleotide Binding Domain (CNBD) whose action is to reduce the inward current during diastole, thus slowing spontaneous frequency and generating bradycardia, with a high correlation between genotype and phenotype. Subsequent work from the PaceLab and other laboratories has in fact revealed the presence of several other arrhythmia-linked HCN4 mutations (167). While most mutations are loss-of-function and are associated with bradyarrhythmia, the author's lab has also found a gain-of-function HCN4 mutation in a family with Inappropriate Sinus Tachycardia (119). These data and others confirm the notion that increasing/decreasing the contribution of I_f to diastolic depolarization accelerates/slows cardiac rate, respectively, in agreement with the functional role of the funny current in the control of heart chronotropism. The data also demonstrate the existence of a broad mechanism for rhythm disturbances based on constitutive alterations of funny channels.

In much the same way as in relation to cardiac arrhythmias, also in neurons, dysfunctional HCN channels can impair excitability and are associated with various neurological diseases, among which epilepsy, neuropathic pain, Parkinson's Disease and even psychiatric diseases such as autism, anxiety, depression (reviewed in DiFrancesco & DiFrancesco, 2015 ref. 170). In collaboration with a team of neurologists, the author has contributed to investigation of cases of HCN-related epilepsy (102, 125, 126, 127, 128).

1) Pharmacological application

It is long known that cardiac diseases such as angina, ischaemic heart disease and heart failure benefit from the slowing of heart rate. It is also known that high heart rate is associated with increased morbidity/mortality in some pathological conditions such as hypertension, myocardial infarction, diabetes etc. Classical agents prescribed to slow heart rate (β- blockers and Ca^{++} antagonists) have potentially adverse side effects (such as for example reduced inotropism) that limit their use. Given the specific role of the funny channel in the generation of spontaneous activity and heart rate control, it may obviously represent a target for the development of drugs aimed to specifically control heart rate, hopefully with limited side effects.

Drug companies have indeed long been seeking for drugs specifically acting on I_f, and only one such drug (ivabradine, developed by Servier) has so far reached the market. The author's PaceLab has contributed a number of publications on the mechanism of action of funny-channel blockers and more specifically on the properties of ivabradine blockade of funny/HCN4/HCN1 channels (72, 87, 108).

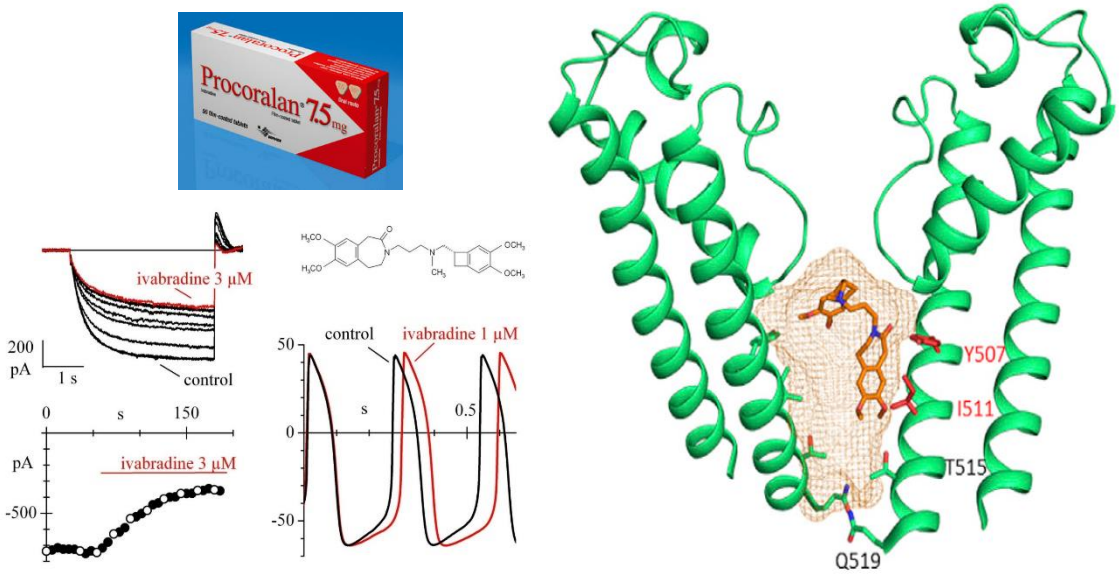

Fig. 8. Left, ivabradine inhibits I_f in an isolated SAN pacemaker myocyte thus reducing the steepness of diastolic depolarization, hence slowing rate. Right, docking of ivabradine to HCN4 channels occurs in the cavity below the pore through interaction with specific S6 residues, as visible in Cryo-EM experiments. From Saponaro et al. 2021 (ref. 133).

Ivabradine is marketed as the "first selective and specific I_f inhibitor" and is successfully used in the therapy of Coronary Artery Disease (CAD) and heart failure (147, 157). Its action directly validates the contribution of I_f to control of diastolic depolarization rate and cardiac frequency. Electrophysiological and molecular studies have elucidated the mode of action of the drug. Ivabradine enters the HCN4 channel pore from the cytoplasmic side and reaches an energetically stable position in the water-filled cavity located below the channel pore (108), where it interacts with specific residues of the S6 domains whose side chains point towards the inner cavity. These interactions have been first identified by docking simulation experiments using an HCN4 homology model (Bucchi et al 2013, ref. 108) and have then been nicely confirmed in a molecular investigation using a Cryo-EM- resolved structure of HCN4 channels (Saponaro et al. 2021, ref. 133).

In conclusion, the original discovery of the funny current, and the subsequent large number of detailed studies of the mechanism of cardiac pacemaking and rate modulation, contributed to by the author and his PaceLab have provided, across different levels, new means to improve the understanding of the cellular processes controlling such a fundamental function of the heart.

While the physiological concept of funny channel-based pacemaking and autonomic rate regulation is now well established, in more recent years clinically-relevant applications of this concept in the heart have been developed, contributing to new fields of research such as biological pacemaking, genetics of arrhythmias, pharmacology of heart rate control.

Funny channels are also highly expressed in neurons, where the same gene product has a variety of different functions. Defective HCN channels are associated with cardiac arrhythmias and, in the brain, with a number of neurological diseases such as epilepsy and others.

Finally, functional expression of HCN channels has been reported to be present in many other other tissues (smooth muscle, embryonic stem cells, leucocytes and more where the role of the funny current is still under investigation (Benzoni et al., 2021). Progress in the knowledge of HCN properties and function will be important to develop new strategies for therapeutic intervention in different diseases.

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