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JMCC Point-Counterpoint:

What keeps us ticking, a funny current, a Calcium clock, or both?

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Prologue

The processes underlying the initiation of the heartbeat, whether due to intracellular metabolism or surface membrane events, have always been a major focus of cardiac research.

About 50 years ago, pioneering work initiated by Silvio Weidmann and others applied the Hodgkin-Huxley formalism of membrane excitation to interpret the cardiac electrical activity, including the pacemaker depolarization (see D. Noble, 1979 The initiation of the heartbeat, Clarendon Press). The underlying idea was that voltage- and time-dependent gating of various surface membrane channels not only generated the cardiac pacemaker action potential (AP), but also controlled the spontaneous depolarization between AP's and thus determined when the next AP would occur. According to this description, the ensemble of surface membrane ion channels works as a clock that regulates the rate and rhythm of spontaneous AP firing, otherwise known as normal automaticity. A formidable research effort then concentrated in attempting to target which of the surface membrane ion channels had an important role in controlling the spontaneous diastolic depolarization (DD).

Originally, a major role was attributed to the "I_K-decay theory". This was strongly influenced by the previous Hodgkin-Huxley model of nerve AP, which described the slow depolarization following a nerve AP as due to the decay of a K^+ current. This model of pacemaker depolarization lasted some 20 years, until it was turned upside-down by a full re-interpretation based on the discovery of the I_f current. Other ionic currents gated by membrane depolarization, i.e. I_{CaL}, I_{CaT}, I_{ST}, non-gated and non-specific background leak currents, and also a current generated by the Na-Ca exchange (NCX) carrier, were also proposed to be involved in pacemaking. Based on a wealth of experimental evidence, I_f is today considered as the most important ion channel involved in the rate regulation of cardiac pacemaker cells, and is sometimes referred to as "the pacemaker channel."

Several studies, some of which recent, have also shown that in addition to voltage and time, surface membrane electrogenic molecules are strongly modulated by Ca^{2+} and phosphorylation. The studies of a sub-group of pacemaker cell researchers focusing upon intracellular Ca^{2+} movements in pacemaker cells spawned the idea that intracellular Ca^{2+} is an important player in controlling pacemaker cell automaticity. This elevated the status of NCX current as a major Ca^{2+}-activated electrogenic mechanism. But the fine details of intracellular Ca^{2+} movements,

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specifically those beneath the cell membrane during DD, were not at hand, and the concept of Ca^{2+} involvement in pacemaking stalled, whilst the concept of I_f control continued to soar—expanding to the design of novel drug development and biological pacemakers.

More recent discoveries over the past decade, made possible by simultaneous submembrane Ca^{2+} imaging and membrane potential or current recordings with cell-attached patch electrodes, have shown that critically timed Ca^{2+} releases occur during the DD and activate NCX, causing the late DD to exponentially increase, driving the membrane potential to the threshold for the rapid upstroke of the next AP. Such rhythmic, spontaneous intracellular Ca^{2+} cycling has been referred to as an "intracellular Ca^{2+} clock", i.e. a component that interacts with the classic sarcolemmal membrane voltage clock to form the overall pacemaker clock.

Needless to say, there is presently some degree of uncertainty about the relative roles of I_f *vs* that of intracellular Ca^{2+} cycling in controlling the normal pacemaker cell automaticity. The dialogue that ensues aims to present and refute both sides of the issue. Sit back and enjoy the show!

Point: DiFrancesco

1. Introductory Note

The Munich theatre of Messe-Munich sits over 3000 and was crammed with sitting and standing delegates when during the last 2008 ESC Congress, Kim Fox, Chairman of the Executive Committee of the BEAUT*I*$_f$UL trial, announced the long awaited results of this international, randomized, double-blind, placebo-controlled study (see http://www.beautiful-study.com) involving 781 centers from 33 countries and enrolling 11,000 coronary artery disease (CAD) patients with left ventricular (LV) dysfunction in the aim to assess cardiovascular prevention benefits of ivabradine, a pure heart rate-reducing agent [1].

The reason for mentioning the BEAUT*I*$_f$UL trial is not to discuss its outcome, but its scientific basis. The strong interest in the performance of ivabradine is due to its unique properties. Ivabradine is the first commercially available molecule able to slow heart rate specifically, without affecting other cardiovascular parameters. This is a consequence of its selective inhibitory action on the funny (I_f) current (see below point 8). Neither the strong interest of clinicians nor the considerable investments made for drug development would make sense if not for a basic fact, verified not just *in vitro* but *in vivo*, on a large population of CAD patients: funny channels drive pacemaker activity and control heart rate, and their inhibition slows heart rate specifically.

2. Basic properties of the funny current

The properties of funny channels are indeed specifically apt to generate the diastolic depolarization phase of the action potential, which is the phase responsible for normal spontaneous activity. This was apparent since the current was first described in 1979 in the sinoatrial node (SAN) [2]. I_f activates upon hyperpolarization, one of the unusual features which at the time of its discovery made the current deserve the attribute "funny", at a threshold of about −40/−50 mV, and is fully activated at about −100 mV. In its range of activation, which quite properly comprises the voltage range of diastolic depolarization, the

current is inward, its reversal occurring at about −10/−20 mV. This is due to the mixed Na^+/K^+ permeability of f-channels [3–6].

The job of an inward current activating on hyperpolarization is to initiate a depolarizing process in the voltage range of activation. At the end of the repolarization phase of an action potential, since I_f activation occurs in the background of a decaying outward (K^+ time-dependent) current, current flow will quickly shift from outward to inward, giving rise to a sudden reversal of voltage change (from repolarizing to depolarizing) at the maximum diastolic potential (MDP). Thus, the basic kinetic and ionic properties of I_f are by themselves a first direct indication of the involvement of this current in generation of the diastolic depolarization and spontaneous activity.

3. Reinterpretation of i_{K2} and formulation of an unified cardiac pacemaking mechanism

Before the I_f current was discovered in the SAN, cardiac pacemaker depolarization (in Purkinje fibres) was thought to arise from a different process, the decay of an outward current (K^+-conductance decay hypothesis). Early investigation of the diastolic depolarization in Purkinje fibres, based on membrane conductance measurements, led to the hypothesis that this process was due to the decay of the delayed K^+ conductance activated during the preceding action potential [7, 8]. Apparently incontrovertible confirmation of this hypothesis came with the description in 1968 by Noble & Tsien [9] of a "pacemaker" pure K^+ current (I_{K2}) which activated on depolarization in the diastolic range of voltages in Purkinje fibres. I_{K2} was described as a pure K^+ current mainly based on evidence for current reversal near the K^+ equilibrium potential. The importance of I_{K2} in pacemaking was amplified by evidence of its involvement in rate acceleration caused by sympathetic stimulation [10]. The “K^+-conductance decay” hypothesis appeared therefore to stand on solid experimental ground, and for over 10 years I_{K2} was considered as the best described cardiac K^+ current, although some of its properties were unexplained; for example, the current disappeared in low external Na^+ solutions [11], and its reversal potential was too negative for a pure K^+ current [12]. The discovery in 1979 of I_f in the SAN introduced a novel mechanism, operating in exactly the opposite direction as I_{K2} (i.e. an inward current activated on hyperpolarization, as opposed to an outward current activated on depolarization) to explain essentially the same process of pacemaker generation, and posed therefore a puzzle: was it really possible that pacemaking in two distinct, but connected regions of the heart, was so dramatically different and based on exactly opposite mechanisms? It was a paradox, and the puzzle was solved only 2 years later, by careful re-evaluation of the I_{K2} current in Purkinje fibres [13][14]. This study showed that the original I_{K2} interpretation, hence the K^+-current decay hypothesis, were incorrect. The Purkinje fibre pacemaker current was not an outward current activated on depolarization, but no less than just the opposite, an inward current activated on hyperpolarization, identical to the SAN I_f. The main reason for the incorrect interpretation was a “fake” reversal potential, which happened to be close to the expected K^+ equilibrium potential, and which was caused by superimposition during hyperpolarizing steps of two components: the activating I_f and a large inward-decaying K^+ current (I_{K1}) [15]. Block of I_{K1} by Ba^{2+} removed the inward-decaying component and left only I_f, thus unmasking the real inward nature of the Purkinje fibre's pacemaker current and allowed for the first time to visualise the "conversion" of I_{K2}

into an inward, hyperpolarization-activated current. These data thus showed that I_{K2} was a "camouflaged" I_f [13]. The demonstration that the two "pacemaker" currents in the two cardiac tissues were of identical nature strengthened the view that I_f activation represents a general mechanism for pacemaker generation in cardiac cells.

4. Autonomic control

When first described in the SAN [2], I_f appeared not only to be involved in the generation of diastolic depolarization, hence of spontaneous activity, but also in the adrenergic modulation of heart rate. I_f indeed increased substantially in the presence of adrenaline (0.1 μM), a change expected to cause acceleration of the diastolic depolarization, hence of spontaneous rate, as experimentally observed.

This finding made sense. If the funny current was responsible for generating diastolic depolarization, then it was reasonable to assume that it could be an useful tool also to *modify* spontaneous frequency. I_f provided therefore not only a means to generate pacemaking, but also importantly to modulate heart rate, and specifically to mediate the positive chronotropic action of sympathetic stimulation.

As it was later found [16], and in agreement with data on the I_{K2} current before its re-interpretation [10], adrenaline increases I_f by a depolarizing shift of the activation curve, which increases current availabilty at all voltages in the activation range.

The role of I_f in pacemaking received further support with the finding a few years later of the muscarinic modulation of I_f. These studies showed that I_f, as well as being activated by β-adrenergic receptor (β-AR) stimulation, is also strongly inhibited by adenylate cyclase (ACh) [17][18, 19]. ACh has an action opposite to that of catecholamines, i.e. inhibits I_f by shifting its activation curve to more negative voltages, which leads to a decreased current availability during diastolic depolarization, hence to rate slowing.

The finding of the ACh-dependent I_f inhibition had a strong impact on the physiology of heart rate regulation since it challenged the universally accepted view that vagal-induced rate slowing results from activation of an ACh-dependent K^+ current ($I_{K,ACh}$) [20]. Investigation of the ACh action on I_f and $I_{K,ACh}$ in SAN cells with protocols where the two currents were simultaneously recorded showed that much lower concentrations of ACh are required to inhibit I_f than to activate $I_{K,ACh}$, and that ACh concentrations inhibiting I_f without activating $I_{K,ACh}$ (0.03–0.3 μM) are perfectly capable of inducing substantial slowing [21]. This was the first demonstration that ACh-induced I_f inhibition, more than K^+-current activation, is involved in rate slowing by moderate vagal activity.

Cyclic adenosine monophosphate (cAMP) mediates the autonomic modulation of f-channels. β-adrenergic and cholinergic neurotransmitters modify the degree of activation of f-channels, hence the size of inward current flow during diastole and steepness of diastolic depolarization, by increasing and decreasing, respectively, the activity of adenylate-cyclase and intracellular cAMP [17–19, 21]. How does cAMP work on funny channels? Investigation in inside-out patches of SAN cell membranes led to the unexpected finding that cAMP works by direct binding to the channel, rather than by cAMP-mediated

phosphorylation [22]. It was unexpected because cAMP-dependent modulation of other ion channels, such as L-type Ca^{2+} channels, was known to occur via phosphorylation [23]. These data demonstrated for the first time the similarity, confirmed years later when Hyperpolarization-activated, Cyclic Nucleotide-gated (HCN) channels were cloned (see below point 6), between f-channels and another class of channels, the cyclic nucleotide-gated (CNG) channels of sensory neurons, which are activated by cyclic nucleotides (even if not by voltage).

The presence of a direct, phosphorylation-independent I_f activation by cAMP suggests a fast cellular pathway for pacemaking control. Another important feature more recently described is that f-channels of SAN cells are compartmentalized and concentrated in caveolae, membrane microdomains where proteins involved in a given signal transduction pathway assemble together [24]. Interestingly, membrane caveolae also contain a high concentration of β2-adrenergic receptors (β2-ARs), which in the SAN are generally expressed at much higher levels than in working myocardium, while β1-ARs are mostly excluded from caveolae [25, 26]. In accordance with these data, in SAN cells pacemaker channels co-localize with β2-ARs in caveolae, and are therefore activated more efficiently by β2 than by β1 stimulation [24]. Compartmentation thus ensures a highly efficient cAMP-dependent modulation of f-channels. Together, direct f-channel activation by cAMP and compartmentation contribute to a well-tuned system for control of f-channel function. The picture that emerges is therefore that of a *rapid* and *efficient* coupling between autonomic stimuli and heart rate, mediated by a cAMP-I_f –dependent mechanism.

5. Correlating I_f and pacemaker activity/pacemaker rate

If the I_f current is really responsible for generation of spontaneous activity and rate control, then expression of f-channels should correlate with pacemaking and rate. Early criticisms of f-channel-based pacemaking relied upon data according to which I_f was too small, or activated at too negative voltages for significant contribution [27]. However it has become evident that I_f run-down during recording from single cells plays an important role in underestimation of the size of the current [16] [22].

Current run-down may be due to the loss of a cytoplasmic factor necessary for correct f-channel function [16]; this situation is exemplified by the strong negative shift which the f-channel activation curve undergoes as soon as membrane patches are excised from the cell to form inside-out patches [28]. This cytoplasmic factor could for example be PIP2 (phosphatidylinostiol 4–5 bisphosphate), since as has recently been shown, it strongly activates I_f by shifting the current activation curve to more positive voltages [29].

As is discussed above, evidence for a correlation between the extent of I_f activation and rate is intrinsic of the basic properties of the current, since a positive/negative shift of the I_f activation curve, i.e. more/less current during diastole, is associated with rate acceleration/slowing. Further evidence supporting this correlation from different perspectives is discussed below.

6. Uniqueness: funny/HCN channels are expressed/functional only in (cardiac) pacing tissue

Of the four HCN α-subunits which contribute to mammalian native funny channels, HCN4 is the dominant isoform in cardiac pacemaker tissue. In the same tissue HCN1 and/or HCN2 isoforms are expressed at lower levels, while HCN2 is more highly expressed (at messenger level) in the ventricle [30, 31] [32]. An indication of the correlation between funny channels and cardiac pacemaking is the evidence that pacemaking tissue and tissue expressing HCN channels largely coincide; in other words, HCN expression is a specific feature of tissue/cells with pacemaker capability. This is particularly apparent in the SAN, where expression of HCN4 is typically concentrated in the central node, and decreases when approaching the peripheral nodal area, eventually to disappear in surrounding atrial regions [31, 32]. HCN4 is therefore considered a specific marker of SAN pacemaker tissue [30–34].

A tight correlation between I_f and pacemaking is also apparent during development. I_f is recorded in spontaneously beating fetal and neonatal ventricular myocytes and disappears from these cells at birth, when ventricular myocytes lose their spontaneous activity [35] [36]. Loss of spontaneous activity and of I_f are well timed and occur in concert [37] [36].

HCN4 expression is specifically stimulated in SAN tissue by T-box 3 (Tbx3), a transcriptional repressor whose activation is essential for development of the SAN and atrioventricular node (AV) bundle and segregation of pacemaker SAN from atrial cell lineages; this correlation further supports the view that HCN4 is a SAN marker gene [38].

HCN2 RNA message, on the other hand, is higher in the adult ventricle than in the SAN [30], but the low level of protein expression [39] along with the highly negative activation threshold [30] make this isoform non functionally active under physiological conditions. Interestingly however, I_f expression is strongly upregulated in pathological conditions, and more specifically in cardiac hypertrophy and heart failure [37] [40] [41] [42]. The increase in I_f expression, mainly due to HCN2 (and HCN4) upregulation, may lead to increased spontaneous cellular activity and thus contribute to ectopic rhythm [43], again supporting a correlation between f-channel expression and pacing.

7. HCN4 functional defects lead to arrhythmias

If a correlation exists between funny current and pacemaker generation and modulation, then one should expect that channel mutations leading to a functionally defective I_f can be responsible for rhythm disturbances. To investigate this, different laboratories have performed genetic screening of individuals with inheritable cardiac arrhythmias affecting sinus rhythm, such as Sick-Sinus Syndrome (SSS), sinus bradycardia and Inappropriate Sinus Tachycardia (IST). A few specific HCN4 channel mutations linked to rhythm disturbances have indeed been identified. Early work investigating this link was based on a single patient [44] or reported a complex arrhythmic behavior which could not be associated with certainty to an HCN4 mutation [45], and provided therefore only suggestive evidence, but more recent data have clearly demonstrated a correlation between defective HCN4 and arrhythmia. For example, mutation to arginine of serine 672, a highly conserved residue in

the cyclic nucleotide-binding domain (CNBD) of hHCN4, was shown to cause inherited asymptomatic sinus bradycardia in a large Italian family spanning three generations [46].

Heterozygous individuals carrying the S672R mutation in this family had an approximately 29% slower rate than the wild-type individuals from the same family (52.2 *vs* 73.2 bpm). The two-point LOD score for this family was very high (5.45), indicating tight linkage between mutation and bradycardic phenotype [46]. Heteromeric wild-type/S672R channels expressed in human embryonic kidney 293 (HEK293) cells to mimic the situation for mutated individuals had an I_f activation curve shifted by about 5 mV to more negative voltages, in comparison to wild-type channels. This shift is appropriate for the rate slowing measured [6]. This was the first evidence for inherited bradycardia resulting from a loss-of-function mutation of hHCN4 whose functional effect could be identified, measured and verified to be quantitatively adequate. Another single hHCN4 mutation was later reported to be associated with familial asymptomatic sinus bradycardia [47]. The mutation G480R occurs in the pore domain and functional studies suggest another type of loss-of-function mutation causing decreased synthesis and trafficking of the channel.

As a whole, these data lend further support to the idea that funny channels control heart rate, and confirm that functionally defective channels can lead to arrhythmias.

8. Ivabradine: specific block of I_f slows rate

The relevance of I_f to pacemaking and rate control makes the current an obvious target for *specific* pharmacological control of heart rate, free of other cardiovascular side-effects. The expectation –if I_f is *really* important in pacemaking- is in fact that drugs able to interact specifically with funny channels should only affect rate by modifying the diastolic depolarization phase of the action potential, and leave unaltered shape and duration of the action potential, as well as the inotropic state of the heart, due to lack of action on other ionic components such as K^+, Ca^{2+} and Na^+ currents.

This expectation has been fulfilled with the development of a family of drugs, the pure "heart rate inhibitors", of which ivabradine is today the only commercially available member. The perspective of developing side effect-free heart rate-slowing agents has long raised the keen interest of drug companies, in the light of limitations in the use of Ca^{2+} antagonists and β-blockers.

The first drug to be developed as a “pure bradycardic agent" was alinidine (ST567), a derivative of clonidine [48]; other agents were subsequently developed, such as falipamil (AQ-A39) and its congener UL-FS49 and, more recently, ZD7288. Heart-rate inhibitors were for some time thought to act mostly as Ca^{2+} channel inhibitors [49], and only based on detailed investigation found to act instead through I_f inhibition [50] [28].

Among the rate-limiting agents more recently developed, ivabradine is the only one having passed all clinical tests, and is today prescribed for therapeutic use against chronic stable angina. Ivabradine blocks funny channels with a high degree of selectivity and use-dependently [51], and slows heart rate by acting selectively on the steepness of diastolic depolarization, with no cardiovascular side effects [52].

Taken together, these results show that substances able to bind specifically to and inhibit funny channels can be used as pharmacological tools for heart rate reduction. The fact that specificity of I_f inhibition translates into specificity of action on rate, and lack of side effects on other cardiovascular parameters, confirms the highly selective functional role of funny channels in generation of pacemaker activity and rate control.

9. Biological pacemaker: transfer of HCN channels leads to pacing

Finally, while data discussed above show that the properties of funny channels can be exploited to induce a controlled *loss-of-function* type of effect, i.e. heart rate slowing, it is natural to ask if the opposite also occurs. Can an HCN *gain-of-function* type of effect induce rate acceleration, or even start pacemaker activity in quiescent tissue/cells? Several results have now been gathered which confirm this hypothesis. Early attempts to investigate *gain-of-function* effects showed that *in vitro* overexpression of HCN2 channels steepens the diastolic depolarization and approximately doubles the rate of spontaneously beating neonatal ventricular myocytes [53]. Overexpression of HCN channels, however, can not only accelerate the rate of pacing cells, it can also *induce* spontaneous activity in resting cardiomyocytes. This is the basis for development of "biological pacemakers", devices whose aim is to eventually replace electronic pacemakers; interest in these novel techniques has grown remarkably in the last few years [54, 55]. Protocols for inducing pacemaker activity include local viral infection with HCN channel genes [56–58], the implant of mesenchymal stem cell engineered to overexpress HCN channels [59], and transfer of embryonic stem cells differentiated towards the cardiac pacemaker phenotype [60]. These approaches have been successful in *in vitro* studies as well as in *in vivo* studies, providing "proof of concept" that transfer of HCN channels is a viable method for pacing ventricular muscle [61]. Thus, increased expression of funny channels is a simple and safe approach to generate repetitive spontaneous activity in normally quiescent cardiomyocytes, again in agreement with a basic pacemaking role of I_f.

REBUTTAL: Lakatta

Dario, you have covered a lot of ground in attempting to establish your POINT: that funny channels drive pacemaker activity and control heart rate. My comments are keyed to the specific sub-points 1–9 of your argument.

1. Points 1 and 8 (Ivabradine)

> **“Neither the strong interest of clinicians nor the considerable investments made for drug development would make sense if not for a basic fact, verified not just *in vitro* but *in vivo*, on a large population of CAD patients … funny channels drive heart rate and control heart rate.”**

Dario, I do not understand how the issues you raise about Ivabradine directly address your POINT that funny channels drive pacemaker activity and control heart rate. The development of a drug that specifically blocks I_f at low doses and reduces heart rate by six beats/min surely does not mean that I_f is the primary pacemaker mechanism. Furthermore, the development of this drug was catalyzed by sustained dogma that I_f current is the primary pacemaker mechanism. In my opinion, the continued promulgation of the idea that I_f is a

dominant pacemaker channel may have indirectly suppressed thought and drug development targeting other, necessary and sufficient pacemaker mechanisms.

2. Basic properties of the funny current

> **"If activates at a threshold of about −40/−50 mV… the basic kinetic and ionic properties of I_f are by themselves a first direct indication of the involvement of this current in generation of the diastolic depolarization and spontaneous activity."**

Dario, I don't think that the basic kinetics and ionic properties of I_f to which you refer tell the whole story about the potential role of I_f in generation of spontaneous activity. In human sinoatrial node cells (SANC), half—maximal voltage for I_f activation is approximately 20 mV more negative, and fully activated I_f conductance is 3–4 times smaller than that typically observed in rabbit SANC [62]. Given the slow kinetics of I_f activation [15], the extent to which I_f becomes activated during DD in primary SANC must be low, in general, but especially in humans. And, in mouse SANC I_f starts to activate at −70 to −80 mV, i.e. more negative than MDP [63], and therefore unlikely even to emerge during spontaneous beating. Although there is no doubt that I_f participates in the generation of the diastolic depolarization in some species (e.g. rabbit), numerous experiments have firmly established that no pacemaker cells stop beating spontaneously when I_f is blocked [64–67], suggesting that I_f is not essential for cardiac pacemaking. Furthermore while genetic knock-out of I_f in adult mice, either global or cardiac conduction tissue-specific, effects some rhythm disturbance (see below), the average resting heart rate is not markedly affected [68, 69]. (A caveat in this regard, however, is that a proper assessment of the intrinsic heart rate in situ, i.e. that rate reflecting intrinsic SA node automaticity, requires complete blockade of autonomic input. But this has not been done so far, to my knowledge.) Anyway, if I_f were indeed "the pacemaker current", more severe consequences of its knockout would be expected.

> **"The job an inward current activating on hyperpolarization is to initiate a depolarizing process in the voltage range of activation."**

Yes, but there are diverse opinions regarding which current initiates the depolarization process in SANC [27]. I_f is not the only current which can initiate the DD process in SANC. For example, some investigators support the idea that I_{Kr} decay plays the crucial role in the DD initiation because suppression of I_{Kr} stops spontaneous activity. Furthermore, initiating the DD by any current doesn't indicate to what extent that current follows through to contribute to the remainder of the DD. Newer evidence in SANC points to a substantial impact of another current on the late DD, i.e. the sodium-calcium exchange current (I_{NCX}) activated by submembrane spontaneous rhythmic local Ca^{2+} releases from sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs). Simulations of a novel pacemaker model that portrays the pacemaker cell clock as a robust system of intracellular and surface membrane proteins [70], emphasizes the critical importance of this diastolic Ca^{2+} releases to normal SANC automaticity.

3. Reinterpretation of i_{K2} and formulation of an unified cardiac pacemaking mechanism

"The demonstration that the two "pacemaker" currents in the two cardiac tissues were of identical nature strengthened the view that If activation represents a general mechanism for pacemaker generation in cardiac cells."

From the outset of its discovery, numerous objections have been raised about the significance of the role of I_f in pacemaker function [64–67], and other researchers in the field have preferred I_{st}, I_{CaL}, I_{Kr}, or I_{NCX} as dominant factors in pacemaker function [71]. Further, I_f is not ordinarily observed in spontaneously beating SANC isolated from monkey and rat [72] and this clearly contradicts the idea that I_f is the general mechanism of cardiac pacemaking. In contrast, other (non-I_f) mechanisms, e.g., intracellular Ca^{2+} cycling and I_{NCX} (Fig. 1), in primary pacemaker cells are conserved both ontogenetically [73–75] and phylogenetically (e.g., Ca^{2+} cycling is important in amphibian toad pacemaker cells [76]) and, accordingly, have achieved the status of general mechanisms. Coupled oscillators, in general, and Ca^{2+} cycling coupled to membrane function, in particular, are universally observed throughout nature in robust oscillators of different tissues and species [77–82]. So, I don't get your message here regarding the uniqueness of I_f based upon just its presence in two cardiac tissues.

4. Autonomic control

"I_f increased substantially in the presence of adrenaline. This finding made sense. If the funny current was responsible for generating diastolic depolarization, then it was reasonable to assume that it could be a useful tool to *modify* spontaneous frequency."

The cornerstone of your argument over the years has been that a shift in I_f activation that occurs following G protein-coupled receptor stimulation, i.e., β adrenergic or muscarinic receptors (or application of cyclic nucleotides or of other signaling molecules) is the cause of the resultant change in the pacemaker firing rate. It is well-documented, however, that other cell signals that you did not embrace are also activated by cAMP. Specifically, protein-kinase A (PKA)- and calmodulin kinase II (CaMK II)-dependent activation are direct or indirect events occurring down stream of cAMP, and Ca^{2+} cycling protein phosphorylation by both kinases impacts on both the timing and amplitude of rhythmic, submembrane Ca^{2+} releases during DD, impacts on sarcolemmal ion channel flux, and impacts on the AP-induced Ca^{2+} transient (Fig. 1). In other words, the demonstration that I_f activation shifts under voltage clamp does not prove that this effect on I_f is either necessary or sufficient to mediate the observed chronotropic effect of autonomic receptor stimulation in intact cells, due to concomitantly occurring other potent downstream intracellular effects of cAMP (Fig. 1).

The most convincing method to prove your POINT about a crucial role of I_f in autonomic heart rate regulation would be to demonstrate that I_f is indeed uniquely required. In other words, to demonstrate that when I_f is disabled the expected effects of autonomic receptor stimuli to alter the spontaneous firing rate of SANC do not occur, or are markedly impaired. But numerous experiments [66, 68, 69, 84–86] utilizing pharmacologic, genetic and even cardiac specific genetic manipulations of I_f, however, demonstrate that robust chronotropic

effects of β-adrenergic receptor (β-AR) stimulation remain intact when I_f is inhibited. Similarly, the full effect of cholinergic receptor stimulation to slow the spontaneous AP firing rate is preserved in the presence of I_f blockade [87, 88]. It may be concluded from this evidence that either the shift in I_f activation is not required, i.e. is not critical to the autonomic regulation of the sinoatrial node cell spontaneous firing rate, or that powerful redundant mechanisms that transducer G protein receptor stimulation signals exist: when one mechanism, e.g. I_f, is knocked out, these other mechanisms (Fig. 1) are sufficient to preserve the chronotropic responses. This later interpretation, however, necessitates the admission that I_f is a redundant mechanism for autonomic responses, and this admission, ipso facto, negates the viewpoint that a shift in I_f activation kinetics is the primary mechanism that controls autonomic responses, and renders this view untenable.

On the other hand, PKA-dependent phosphorylation effects on SR Ca^{2+} cycling, local intracellular Ca^{2+} levels and CAMKII activation each markedly increase following an increase in cAMP in response to β-AR stimulation (Fig. 1). Furthermore, when ryanodine receptors are disabled pharmacologically [85, 89, 90] or genetically [91, 92], or PKA activation is prevented, spontaneous submembrane local Ca^{2+} releases (LCR's) do not occur during the DD (even when an AP-triggered Ca^{2+} transient still occurs) [91, 92], and the ability of the β-AR or cAMP to accelerate SANC firing is markedly reduced. These results clearly differ from genetic or pharmacologic I_f inhibition, and indicate that PKA, Ca^{2+} or CAMKII-mediated events, unlike I_f, are not redundant with other mechanisms, and must indeed be necessary control points for translating autonomic receptor signals. Furthermore, the failure of the β-AR chronotropic response when these PKA dependent mechanisms are knocked out indicates that the shift of I_f activation in response to a change in cAMP is not sufficient to effect a normal chronotropic response.

"The presence of a direct, phosphorylation-independent I_f activation by cAMP suggests a fast cellular pathway for pacemaking control."

For sure, a rapid-efficient coupling is required for the initiation of robust heart rate modulation. But, Dario, the data on β-ARs and on caveoli that you cite here demonstrate neither rapidity nor efficiency. While β-AR stimulation or phosphodiesterase (PDE) inhibition markedly increases cAMP, I_f current and the spontaneous SANC beating rate, the steady state effects of either β-AR stimulation or PDE inhibition on the beating rate are completely preserved when I_f is inhibited [64, 85, 89]. This indicates that mechanisms other than I_f become activated by cAMP and control the heart rate in response to β-AR stimulation or an increase in cAMP. This other rapid-efficient coupling system involves colocalization of protein kinase anchoring proteins (AKAP's) with ACs to effect rapid PKA activation and signaling upon cAMP formation. PKA, in turn, rapidly affects Ca^{2+} cycling, the LCR period becomes reduced, and LCR's begin to occur sooner following the prior AP-induced Ca^{2+} transient. When this signaling system is experimentally disabled [83, 93], both basal beating and autonomic responses are markedly reduced, and with further reduction in PKA-dependent Ca^{2+} cycling, spontaneous beating ceases.

5. Correlating I_f and pacemaker activity/pacemaker rate

"If the I_f current is really responsible for generation of spontaneous activity and rate control, then expression of f-channels should correlate with pacemaking and rate."

Exactly! But what if I_f does not highly correlate with pacemaking and rate control, but other mechanisms do? Might it then be concluded that I_f current is not really responsible for spontaneous activity and rate control? This, in fact, is the case, because, as already noted, numerous studies demonstrate that blocking I_f pharmacologically [64, 65, 85, 94] has only minor (7%) to moderate (31%) effects on the spontaneous AP firing rate of SANC, but never stops spontaneous AP firing. Similarly, eliminating I_f genetically in adult mice has minor effects on the average heart rate [68, 69]. Doesn't this mean that in adult SANC I_f can hardly be said to "really be responsible for generation of spontaneous activity and rate control" and cannot continue to be portrayed as "The Pacemaker Current"?

In contrast, more modern approaches, using a perforated patch to prevent intracellular dialysis and disruption of subtle, but crucial Ca^{2+} cycling-driven events, have cast a whole new light on an entire constellation of mechanisms that drive pacemaker function and control heart rate that link Ca^{2+} to cAMP and cAMP to PKA-CaMKII Ca^{2+} signaling (Fig. 1). For example, blocking AC activity [93, 95] or chelation of Ca^{2+} [96] stops SANC beating. Submaximal concentrations of ryanodine abolish LCR's and markedly slow the beating rate and disrupt the normal beating rhythm [97]. It might be argued, however, that SANC have Ca^{2+} activated AC's [83, 95, 98], Ca^{2+} chelation or ryanodine may reduce cAMP, resulting in less I_f activation and their effect to interfere with spontaneous beating might be attributable to a reduction in I_f. But, this argument is invalid, because: 1) experimental evidence has proven that direct I_f inhibition has only moderate effects, at best, to slow basal beating compared to the marked effects of Ca^{2+} chelation, or ryanodine to slow basal beating or to prevent a normal chronotropic response to β-AR stimulation; 2) I_f, measured in the absence of Ca^{2+} chelation, is not affected by ryanodine [117]; 3) blunting of the chronotropic response to β-AR stimulation or interfering with Ca^{2+} cycling by ryanodine occurs in the presence of a full cAMP/PKA-dependent augmentation of I_{CaL} [85, 89] suggesting that cAMP-PKA signaling remains fully intact, i.e. I_f is likely also normally activated by cAMP under these conditions. As noted, inhibition of AC mediated effects on intracellular Ca^{2+} cycling that are downstream of cAMP, i.e. PKA, and, indirectly, inhibition CaMKII signaling, effectively slow or stop spontaneous beating [93, 96]. And, of note, cell-permeant cAMP is not effective in accelerating the beating rate when RyRs are disabled by ryanodine (Online Fig. 3 in [93]); this result is in contrast to your earlier study [99]. Importantly, graded changes in PKA-dependent phosphorylation of Ca^{2+} cycling proteins by a specific PKA inhibitor are highly correlated with graded changes in the LCR period, which in turn are extremely highly correlated with graded changes in the cycle length [93]. Furthermore, acute blockade of NCX [97, 100] stops spontaneous SANC beating, leaving LCR's intact [97]. In my opinion, the profound effects on spontaneous beating effected by blockade of these PKA-Ca^{2+}-CaMKII mechanisms that are downstream to c AMP, indicate that these are necessary mechanisms for normal rate control of pacemaker cells.

6. Uniqueness: funny/HCN channels are expressed/functional only in (cardiac) pacing tissue

"HCN expression is a specific feature of tissue/cells with pacemaker capability. This is particularly apparent in the SAN, where expression of HCN4 is typically concentrated in the central node, and decreases when approaching the peripheral nodal area"

It is interesting to note that hearts of mouse embryos lacking HCN4 (the "pacemaker" I_f channel) contract rhythmically, albeit at a reduced (approx. 50%) rate, i.e., "a basal heart rate can be sustained without I_f" [86]). Furthermore, a requirement for I_f cannot be demonstrated in adult pacemaker tissue, because conditional knock-out of I_f, even cardiac specific knock-out, fails to reduce the average resting heart rate [68, 69]. Of note, here, is that knock out of either NCX [101] or RyR [102] in mice is embryonic lethal. Also, more recent studies in embryonic cardiomyocytes show that spontaneous Ca^{2+} cycling rhythmically paces these cells even prior to the expression of HCN [73, 75]. Furthermore, pharmacological blockade of either NCX or RyR, in contrast to I_f blockade, markedly slows or halts spontaneous SANC beating [97, 100]. On the other hand, no one contends that HCN is a marker for pacemaker cells in general; but being a marker doesn't prove control of pacemaking in primary pacemaker cells. In fact, I_f current density is lowest in small size SANC purported to be the primary pacemaker cells [103]. Additionally I_f inhibition has a greater effect in small balls of cells isolated from the periphery compared to the central SAN region [104]. Finally, it has been demonstrated that I_f contributes more to DD in secondary pacemakers (Purkinje cells, subsidiary atrial pacemakers, and AV node cells), which have a lower maximum diastolic potential (MDP) and longer cycle length, permitting a greater availability of f-channels and more time for slow I_f activation, respectively [105].

"HCN4 expression is specifically stimulated in SAN tissue by Tbx3, a transcriptional repressor whose activation is …"

That both Tbx3 and the gene expression it controls are of vital importance to normal heart function during development does not indicate a primary role of HCN4 (I_f) in rate control. In fact, in addition to having some role in rate control, it can be envisioned that I_f also sets the "playing field" for SAN formation and activity, by preventing hyperpolarization from atrial cells. In this sense, I_f could serve a crucial basic housekeeping function.

"When ventricular myocytes lose their spontaneous activity, the loss of spontaneous activity and of If are well timed and occur in concert. "

But, during development changes in other electrophysiological and biochemical mechanisms that affect pacemaking also emerge in ventricular cells, e.g., I_{K1}, amidst differential regulation of other inward currents; and NCX expression decreases [106]. In other words, I_f is not exclusively developmentally regulated in cardiac cells, but is just one of numerous adjustments of mechanisms that are no longer required in cells that are not designed to beat spontaneously any longer [107].

7. HCN4 functional defects lead to arrhythmia

> **"If a correlation exists between funny current and pacemaker generation and modulation, then one should expect that channel mutations leading to a functionally defective I_f can be responsible for rhythm disturbances."**

Recent genetic experiments do indeed indicate that the presence of HCN4 stabilizes the pacemaker rhythm, particularly at slower rates, when HCN channels can become more activated [68, 69, 108] or in transition states following β-AR stimulation [68]. Additionally, pharmacologic inhibition by Ivabradine and its predecessors, at concentrations greater than those producing modest bradycardia clinically, can induce striking dysrhythmias [109]. Such dysrhythmic effects of I_f blockade are indeed predicted by a novel pacemaker model [70] mentioned earlier (cf response to point 2 above). Specifically, the modeled pacemaker system without I_f has substantially smaller parametric space of rhythmic firing [70]. But these are moot issues, since mutations in any surface membrane channel or intracellular protein that contributes to pacemaking, human RyR mutations, for example, also lead to rhythm disturbances and cause severe SAN dysfunction and atrial fibrillation [110].

> **"As a whole, these data lend further support to the idea that funny channels control heart rate".**

My interpretation here differs widely from yours. My interpretation would be that since the human I_f mutations generally produce a mild slowing of heart rate, it cannot be said that I_f controls heart rate, but rather mildly contributes to heart rate regulation, in the context of other necessary and sufficient regulatory mechanisms (Fig. 1). For example, Lithium can produce profound sinus node dysfunction [111].

9. Biological Pacemaker Transfer of HCN Channels Leads to Pacemaking

> **Thus, increased expression of funny channels is a simple and safe approach to generate repetitive spontaneous activity in normally quiescent cardiomyocytes, again in agreement with a basic pacemaking role of I_f.**

i. Simple–yes.

ii. Safe–yet untested.

iii. Flexible--not likely.

iv. In agreement with a basic pacemaking role of I_f.--Yes, a role in, but not in control of, basic pacemaking. I am sure that you would agree, Dario, that genetic transfer of any molecules that generate inward current or block outward currents will impact on spontaneous activity of ventricular cells.

Summary—It is clear to me that, funny channels are not only a marker for SAN tissue, but are also important to development of and the normal function of the SAN in embryo. An important role for funny channels in rhythm stability in adult SANC is emerging, and I believe that this property could be one of the most important functions of I_f and surely merits further study. Furthermore, it is clear that I_f human mutations are, indeed, associated with a mild reduction in resting heart rate, and well-tolerated doses of Ivabradine can provide a mild reduction in resting heart rate in humans. But experimental inhibition of I_f

does not usually markedly reduce the basal beating rate. And most importantly, it has become absolutely clear that cAMP-mediated shifts of funny channel current activation are neither necessary, nor sufficient to account for autonomic modulation of pacemaker cell firing rate. In my opinion, Dario, you have not provided any substantive direct evidence to prove your point that funny channels DRIVE pacemaker activity and CONTROL heart rate. Thus it is difficult for me to share your opinion regarding the supremacy of I_f in pacemaker rate control. In contrast, mechanisms other than I_f, that are distal to cAMP formation (Fig. 1), are both necessary and sufficient for pacemaker cell rate control, because when these mechanisms are inhibited, spontaneous beating markedly slows or stops and robust responses to G-protein-coupled receptor signals are markedly impaired. Thus, it seems to me, that a number of redundant mechanisms INTERACT within a SYSTEM (Fig. 1) to ensure both robust and flexible pacemaker firing rate control. It is essential, in my opinion, that future research on pacemaker function, creation of biologic pacemakers or drug development addresses the pacemaker cell, not as having a dominant molecule for pacemaking, but rather embraces pacemaker cell function as a robust yet flexible system of interacting molecules (Fig. 1) [70, 112].

Counterpoint: Lakatta

The Time Keeping Mechanism of the Heart's Pacemaker Clock: A Modern Perspective

- Spontaneous rhythmic Ca^{2+} cycling via the sarcoplasmic reticulum Ca^{2+} pump and ryanodine receptors is the pendulum of the heart's pacemaker clock

Advances regarding SR pumping and releases via ryanodine receptors (RyR) within ventricular cells have, over the last 20 years [113, 114], dramatically influenced pacemaker cell research [67, 115]. Early studies that had employed the plant alkaloid ryanodine, which locks RyR's in an open sub-conductance state, resulting in depletion of SR Ca^{2+} content and impaired Ca^{2+} release, have been pivotal in this regard [116]. Ryanodine has a profound effect to: reduce the amplitude of SANC action potential (AP) induced Ca^{2+} transient; impair activation of an inward NCX current, flatten the late diastolic depolarization (DD); slow the SANC spontaneous beating rate, caused dysrythmic AP firing and often AP firing [114, 117–124]. There is apparent disagreement that ryanodine equally effects the spontaneous beating rate of isolated SANC of all sizes [124, 125] (but note that the variation about the mean ryanodine effect in small cells in the latter report [125] is greater than the mean effect per se). That blockade of the SR Ca^{2+} pump [100], like the effect of ryanodine, strongly reduces the rate of spontaneous beating of the SAN [119] also implicated Ca^{2+} cycling in normal pacemaker automaticity. A link between diastolic Ca^{2+}, NCX, and the spontaneous firing has also been demonstrated in toad pacemaker cells [123].

Although immunolabeling of SANC RyR's indicated that these were largely sub-sarcolemmal [118, 126], the spatial-temporal characteristics of sub-sarcolemmal Ca^{2+}cycling were lacking, as were specific mechanisms of how this is regulated, and how it relates to normal automaticity. Confocal imaging of Ca^{2+} in SANC combined with a non-invasive perforated patch-clamp electrophysiology during this decade, however, has documented the occurrence of rhythmic, RyR-generated, sub sarcolemmal local Ca releases (LCR's) [97, 127], and explored how specific mechanisms of LCR generation contribute to

normal SANC automaticity [76, 85, 89, 93, 96, 97, 115, 124, 128–130]. LCR's are observed as 4–10 μm Ca^{2+} wavelets; they emerge following a variable delay after the decay of the global cystolic transient effected by a prior AP, and crescendo during the DD, peaking during the late DD, i.e., within 100 msec prior to the next AP [89, 97] During voltage clamp of SANC, LCR's are clearly spontaneous and rhythmic, and generate miniature, current fluctuations due to I_{NCX} activation [128, 130]. The anatomical counterpart of this sub–sarcolemmal RyR-NCX crosstalk is that both molecules are colocalized (at the level of the confocal microscope) [124]. While an initial report [126] indicated week immunolabeling of RyR, NCX, and SR Ca^{2+} pump protein (SERCA) in small SANC and in cells within the center of the SAN, a later report [124] refuted this conclusion. (The reader is urged to refer to compare the robustness of the data in these reports of this controversial issue).

The speed at which the SR cycles Ca^{2+} determines the LCR period [130], which is reported by the duration of time between the onset of the prior AP-induced Ca^{2+} transient and LCR emergence. Analysis of the fine DD structure indicates that the LCR period and amplitude determine time of onset and amplitude of the late DD. Thus, the occurrence and summation of LCR-activated NCX currents is a major cause of the exponential rise of the late DD under basal conditions [128] and a critical determinant of when the threshold for the next AP firing will be achieved. (This exquisite link between the onset of the exponential DD (and its amplitude) and the spontaneous firing rate is not discovered by traditional analytic approaches that do not measure the onset and amplitude of the non-linear DD [131]). The substantial body of experimental evidence, indicating that changes in LCR periodicity, amplitude and spatial width in response to chronotropic perturbations are highly correlated with the evoked concomitant changes in spontaneous cycle lengths across the entire physiologic range [93, 115] gives rise to the notion that rhythmic LCR generation is part of a "Ca^{2+} clock" (Fig. 1) that is crucial to normal automaticity [115]. Simulations of a novel pacemaker model that portray pacemaker clock as a robust system intracellular and membrane proteins [70] substantiate the interpretations of these experimental data.

Regulation of robust basal pacemaker clock function

The LCR period under a given condition reports the kinetics of SR Ca^{2+} cycling, i.e., the Ca^{2+} clock "ticking speed" under that condition. Regulatory factors of the kinetics and amplitude of spontaneous SR Ca^{2+} cycling include the Ca^{2+} available for pumping, and SERCA2 and RyR function, modulated by cAMP-PKA dependent and CaMKII-dependent phosphorylation (Fig. 1). Constitutively active, Ca^{2+}-activated Adenylyl Cyclases (AC types 1 and 8) are expressed in SANC [83, 95] and produce high levels of not only cAMP, but also cAMP-mediated PKA-dependent phosphorylation of proteins involved in SR cell Ca^{2+} balance and SR Ca^{2+} cycling [83, 93]. SANC also have a high level of basal activity of CaMKII [96], and, in saponin-"skinned" SANC, both PKA and CaMKII activation is involved in the generation of spontaneous LCR's and modulation of their characteristics [132, 133]. Thus, regulatory factors of SR Ca^{2+} cycling work as a complex biological system with numerous functional redundancies that include both "feed-back" and "feed-forward" loops that enhance its robust operation: Ca^{2+} binds to calmodulin to (1) activate adenylyl cyclase, leading to cAMP-dependent PKA activation (2). PKA dependent phosphorylation, in turn, enhances Ca^{2+} release, activating CaMKII, which further promotes

Ca^{2+} cycling (Fig. 1); this “feed-forward” signaling, i.e., Ca^{2+} release begets Ca^{2+} release, is kept in check by factors that regulate Ca^{2+} AC, PKA or CaMKII activities so that the Ca^{2+} clock remains stable in a given steady state. High basal phosphodiesterase (PDE) activity within SANC [89] is one such control point: inhibition of basal PDE within SANC markedly elevates cAMP mediated, PKA-dependent -protein phosphorylation of SR and surface membrane proteins (Fig. 1); LCRs occur more frequently and become larger due to recruitment of additional RyR’s [89]. Conversely, a reduction of cAMP via inhibition of AC activity, or direct inhibition of PKA or CaMKII activation, leads to a reduction of phosphorylation of Ca^{2+} clock regulatory proteins, and slowing or abolition of LCR’s [93, 134]. Graded changes in LCR characteristics that occur in response to graded levels of PKA-dependent protein phosphorylation are directly correlated with concomitant increase or decrease in the spontaneous firing rate in response to PDE or AC inhibition, respectively [89, 93].

Sarcolemmal ion channel function and SR Ca^{2+} cycling are intimately coupled during spontaneous AP firing, because the very same regulatory factors, i.e., Ca^{2+}, PKA and CaMKII dependent activation [89, 93, 96], influence both LCRs and surface membrane proteins (Fig. 1). In addition, cAMP directly activates some sarcolemmal ion channel proteins [22] (Fig. 1). Sarcolemmal proteins that regulate SANC Ca^{2+} balance are essential components of the pacemaker Ca^{2+} clock because they regulate the balance of influx and efflux of Ca^{2+} during a cycle that directly sustains a given cell Ca^{2+} level for pumping into the SR that is required to generate LCR’s with stable beat-to-beat characteristics [70, 130]. Sarcolemmal proteins, NCX and I_{CaL}, are not only essential for SR Ca^{2+} clock [97, 100] but are inherent components of the system Ca^{2+} clock that not only sustain the cell Ca^{2+} level, but regulate/tune it commensurate to permit variations in Ca^{2+} clock ticking that underlie variations in the SANC cycle length [70, 115, 129]. L-type Ca^{2+} channels, which, in addition to generating the rapid AP upstroke, synchronize the intracellular SR Ca^{2+} clocks by triggering a global SR Ca^{2+} depletion. In turn, the AP-induced Ca^{2+} release directly inactivates I_{CaL}, i.e., another Ca^{2+} clock control point (Fig. 1). Activation of NCX by LCR’s exponentially accelerates the late DD, promotes I_{CaL} activation, which, prior to rapid AP upstroke, leads to additional Ca^{2+} release during the late DD by I_{CaL}, and possibly by I_{CaT} in species in which this channel is expressed [135], increasing the submembrane compartment $[Ca^{2+}]$, i.e., another feed forward system to ensure the transition from the late DD to the full-blown rapid AP upstroke. There is some evidence to indicate that I_{CaT} activates LCR’s in cat latent pacemaker cells [127], but this is not the case for rabbit SANC [85]. Thus, common regulatory factors of intracellular molecules that influence SR Ca^{2+} cycling and gating of sarcolemmal ion channels mutually entrain these molecules. Such mutual entrainment ensures efficient, tight coupling of membrane and intracellular components of the SANC pacemaker clock, enabling both robust and stable basal state AP firing [115].

Furthermore, regulation of cell Ca^{2+} by sarcolemmal proteins depends not only on events that occur during an AP, but also depends upon the rate and rhythm of AP occurrence: changes in the AP firing rate or AP shape, per se, due to any cause, would be expected to subsequently effect changes in the LCR characteristics, and thus in the spontaneous AP firing rate, via an effect on steady state cell Ca^{2+} levels, i.e., via the Bowditch treppe effect

[136]. In other words, positive or negative chronotropic perturbations not only have various direct specific effects to change the SANC cycle length, but also likely have indirect, feed-forward effects, resulting from the change in AP firing rate, per se, that is effected by these perturbations[70].

In addition to robustness or fail-safe operation, the heart's pacemaker clock must tick not only at a single rate, but over a wide range of frequencies that encompass the physiologic range of heart rates. Early studies in latent pacemakers from cat atrium had indeed suggested that SR Ca^{2+} release and I_{NCX} were involved in the chronotropic response to β-adrenergic (β-AR) stimulation [122]. More recent studies show that this flexibility of the pacemaker clock's "throttle" is achieved via G protein coupled receptor signaling via (yes, you got it) the very same factors that regulate basal state SR Ca^{2+} cycling, i.e., cAMP and cAMP-mediated, PKA-dependent and CaMKII-dependent phosphorylation [89, 93, 132–134] and Ca^{2+} itself (Fig. 1) and couple this Ca^{2+} cycling to the surface membrane electrogenic proteins. Graded changes of lumped LCR signal phase and amplitude that result from graded changes in these coupling factors, in turn, cause gradations in NCX current timing and amplitude, which, in turn, regulates the timing and the strength of the late DD, hence permitting a wide range of stable cycle lengths [70, 115, 129].

β-AR stimulation, like PDE inhibition, specifically increases the LCR integral by increasing the LCR number and synchronizing RyR activation by local recruitment [85, 89]. The LCR period becomes markedly shorter, shifting LCR occurrence to an earlier time during DD, i.e., a time occupied solely by early DD mechanisms during basal state beating [85]. That is why pacemaker rate acceleration is linked to both the early and late DD phases [128, 131]. The increase in spontaneous AP firing rate in response to β-AR stimulation [85], membrane-permeate cAMP [93] or PDE inhibition [89] requires intact SR Ca^{2+} cycling: when SR Ca^{2+} cycling is inhibited by ryanodine, the expected acceleration of the beating rate is markedly reduced, despite the persistence of a marked augmentation of I_{CaL} in response to these interventions [85, 89]. Studies in both intact isolated canine hearts [90] and in intact canines [93] have validated this discovery made in single isolated SANC. Of note, in cardiac cells derived from mouse embryonic stem cells, intact RyR function is also required for both a normal basal spontaneous beating rate and for a normal response to β-AR stimulation [91, 92, 137].

Threshold and low level cholinergic receptor (CR) activation reduces AC-cAMP-PKA signaling in an inhibitory guanine nucleotide protein (G_i) dependent manner, and this accounts, in part, for the CR stimulation induced reduction in beating rate via a decreased coupling of sarcolemmal ion channels and LCRs [88, 138]. At higher CR ligand (carbachol) concentrations (>30nM), I_{KACh} becomes activated, and the integration of G_i activated I_{KACh} and G_i inhibition of AC, leading to reduced PKA-dependent signaling via G_i induction of AC and to reduction in LCRs, I_{CaL}, and I_f (Fig. 1), produces a marked negative chronotropic effect [88, 138].

Modern Numerical Pacemaker Models: SANC are Coupled Oscillators

The discovery and functional importance of LCR's to normal automaticity of SANC has spawned a new breed of novel numerical models [70, 114, 129][128] in which rate

regulation is governed by intracellular Ca^{2+} cycling and its coupling to the surface membrane. This fundamentally differs from classical pacemaker models [71, 139], which attribute the rate regulation of SANC mainly to the I_f activation state and its relation to the early DD slope. The rate and amplitude of SR Ca^{2+} cycling of the most recent model [70] is controlled by the amount of free Ca^{2+} in the system, the SR Ca^{2+} pumping rate and the number of activated RyR's. The LCR period and amplitude determine the time and amplitude of the late exponential phase of the DD, respectively, and thus determine whether the membrane achieves its excitation threshold to generate the next rhythmic APs via activation of I_{NCX}. Concomitant changes in SANC cycle length and LCR period and subspace $[Ca^{2+}]$ generated by the integrated LCR signal that occur in response to changes in the regulatory factors of SR Ca^{2+} cycling and its coupling to the surface membrane or in response to G_i protein coupled receptor signaling that have been observed experimentally, are faithfully simulated by these models.

Summary

On the basis of prolific experimental evidence supported by novel numerical modeling, it is reasonable to conclude that the tightly coupled system of SR Ca^{2+} cycling and surface membrane proteins is the clock that controls SANC normal automaticity, leading to their mutual functional entrainment [115]. The clock is robust because the same factors that regulate SR Ca^{2+} cycling, i.e., Ca^{2+} and PKA and CaMKII dependent protein phosphorylation, also regulate sarcolemmal ion channel function and thereby couple SR Ca^{2+} cycling to the surface membrane. G protein coupled receptor signaling ensures pacemaker flexibility by affecting rate regulation by impacting on the very same factors that ensure pacemaker fail-safe operation or robustness in a given steady state. Intimately intertwined robustness and flexibility of the heart's pacemaker ensure a wide range of stable heart rates.

Rebuttal: DiFrancesco

Ed, you concentrate on the existence of a complex cellular machinery underlying Ca^{2+} cycling. I have no problem with this, but we are discussing here the function of Ca^{2+} cycling and that of the funny current. Clearly, none of the mechanisms working in a pacemaker cell can be functionally removed without major impact on the whole behaviour of the cell. Normal physiological pacemaking depends on the integrity of all participating cellular processes, and pointing out that one mechanism is essential for rate control does not mean that mechanism is responsible for that control. The questions we need to address here are simpler ones: which is the physiological process selected to generate spontaneous activity (or, better, discriminating between pacing and silent cardiomyocytes)? Also, when a change in rate is required, which is the physiological process selected, typically by the autonomic nervous system, to produce it? A change in rate of Ca^{2+} fluctuations, or a change in I_f?

General considerations

Ca^{2+} oscillations occur in a variety of cell types and control many different processes [140]. In heart the function of Ca^{2+} transients is to trigger contraction, a property common to all cardiac myocytes and certainly not exclusive of SAN pacemaker cells. The same set of

processes generating Ca^{2+} cycling is present in all cardiomyocytes, but this is not enough to make all cardiomyocytes pace spontaneously. The funny current, on the contrary, is physiologically expressed only in pacing cells. Seeing this from a developmental point of view, pacing is a generalized property of the embryonic heart, in accordance with evidence that HCN4 is expressed early in the cardiac mesoderm [33]. During early cardiac development, HCN4-positive cells are segregated from the surrounding atrial cells by activation of a specific gene program, whose aim is to delineate the functional development of the conduction tissue [38]. As a result, HCN4 is considered as a unique marker of the developing SAN [33]. Incidentally, the funny current is also expressed in several types of neurons, where one of its functions is pacing.

Ca^{2+} spontaneous oscillations are the natural outcome of the complex set of processes governing Ca^{2+} fluxes across sarcolemma and internal Ca^{2+} stores, certainly a suitable property for a mechanism underlying rhythmic contractions. It is therefore not surprising that Ca^{2+} oscillations occur even in the absence of membrane voltage oscillations. What is really important is that voltage transients and Ca^{2+} transients are mutually entrained. Two oscillatory mechanisms cannot be mutually entrained (in syncrony) if their spontaneous frequencies are vastly different. Suppose for example that the spontaneous rate of Ca^{2+} oscillations were 1/2 that of membrane oscillations: in this case Ca^{2+} stores would not be reprimed quickly enough, and contraction would fail once every two cycles. Your finding that Ca^{2+} cycling has an intrinsic rate close to action potential rate confirms that the two processes are easily coupled.

According to this view, tuning of Ca^{2+} oscillations to action potential rate increases the likelyhood of firing at a given “resonance” frequency. Since entraining must work at different frequencies, factors accelerating/slowing the rate of voltage oscillations must also act similarly on Ca^{2+} oscillations. The need to entrain at all frequencies is how I explain why "sarcolemmal ion channel function and SR Ca^{2+} cycling are intimately coupled".

Voltage and Ca^{2+} oscillations are coupled, but which entrains which? Entraining works both ways, in the sense that each of the two processes affects the other, as suggested by evidence that changes in any of the two mechanisms modifies rate. This is not to say that the two mechanisms have the same physiological role. My view is that the main physiological role of the funny current in heart is to control heart rate, and that of Ca^{2+} transients is to control contraction. This does not in any way underestimate the important role of Ca^{2+} as a second messenger in a variety of other cellular processes.

Acting through NCX, Ca^{2+} transients do indeed affect threshold and timing of action potential firing. In my view however this is not the main mechanism for physiological control of rate. It is instructive for example to compare how exactly I_f and Ca^{2+} transients affect rate and rate modulation. Ryanodine greatly reduces Ca^{2+} transients and simultaneously slows rate of pacemaker cells; slowing, however, is caused by a depolarizing shift of the action potential threshold, a mechanism compatible with a reduced contribution of NCX in the last fraction of diastolic depolarization but quite distinct from the reduced steepness of early diastolic depolarization (EDD) observed during ACh-induced bradycardia. A less steep EDD is caused instead by specific I_f inhibition (by ivabradine).

Thus, I_f inhibition is the main mechanism used by (low concentrations of) ACh to slow rate; similar evidence supports the view that βAR-induced rate acceleration is caused by I_f activation [131]. In Fig. 2 the effects on the action potential shape of ivabradine, ryanodine, isoprenaline and acetylcholine are shown. Clearly the mode of action of isoprenaline and acetylcholine is the same as that of ivabradine, i.e. a change of the slope of DD involving the early part of DD itself, and differs qualitatively from the mode of action of ryanodine, i.e. a prolongation of the late part of DD and a depolarizing shift of action potential threshold, with little or no change in the early fraction of DD.

An observation often made to claim a reduced role of I_f in pacemaking is that I_f block by Cs^+ or other blockers does not stop pacemaking. However, block of I_f by Cs^+ and specific blockers is not a full block. By using a proper fully-activating I/V protocol, it can be shown for example that at −50 mV, over 40% of the current is unblocked in the presence of 5 mM Cs^+ [16]. Furthermore, Cs^+ lacks specificity since it is a known K^+ channel blocker, and its I_f-inhibitory effect at the MDP will be balanced by inhibition of K^+ repolarizing currents, further reducing the slowing caused by I_f block. Ivabradine, like other rate-reducing agents, has a much higher f-channel specificity, but its block is strongly use- and frequency-dependent, since it is strengthened by depolarization and released by hyperpolarization. Ivabradine therefore blocks I_f only partially during activation/deactivation protocols (3 μM ivabraine block about 66%, Fig. 1 of [51].

Finally, it is known that the R4496C mutation of RyR2 receptors causes a malignant, autosomal dominant form of CPVT [141]. Mutant mice carrying this mutation, which leads to substantial increase in SR Ca^{2+} release, have a CPVT phenotype, but the same rate as WT mice [142], ruling against a role of Ca^{2+} transients in rate regulation.

Knock-out of HCN4 in mouse has given variable results. Global HCN4 KO led to lack of SAN development and death at embryonic day 9.5–11.5, with a marked slowing of embryonic rates [86]. More recent data on conditional KO of HCN4 showed the onset of sinus pauses, but no apparent basal rate slowing or impairment of sympathetic stimulation [68, 69]. These results however suffer from an important limitation, in that KO of HCN4 was not cardiac-specific. Since HCN4 is expressed in many tissues, it is impossible to separate cardiac from non-cardiac effects. More reliable data will need the development of inducible and cardiac-specific HCN4 KO animals. Preliminary data in my laboratory indicate that inducible, cardiac-specific HCN4 KO indeed leads to rate slowing and death (not shown).

Specific points

In relation to β-adrenergic modulation, you wrote: "The LCR period becomes markedly shorter, shifting LCR occurrence to an earlier time during DD, i.e., a time occupied solely by early DD mechanisms during basal state beating [85]. That is why pacemaker rate acceleration is linked to both the early and late DD phases [128, 131]".

Ed, this does not work, since whichever the shift to earlier times in LCR occurrence, certainly no change must occur, according to your hypothesis, at times preceding LCR occurrence itself, while there is very clear evidence for DD steepening immediately after the

MDP upon β-adrenergic stimulation (Fig. 6 of [131] and Fig. 2). Even more evidently, this reasoning fails to explain the mode of muscarinic-induced rate slowing, since shifting LCR occurrence to later times during DD should only affect the part of DD following LCR occurrence in control, at times overlapping the control AP or later (which indeed is what happens with ryanodine, Fig. 3 of [131] and Fig. 2); instead, ACh clearly decreases early DD slope, like ivabradine (Fig. 7 of [131] and Fig. 2).

You wrote: "Of note, in cardiac cells derived from mouse embryonic stem cells, intact RyR function is also required for both a normal basal spontaneous beating rate and for a normal response to β-AR stimulation[91, 92, 137]".

Ed, again no one denies the importance of mantaining the integrity of all cell processes for correct pacemaker generation and autonomic control of rate, but this does not mean Ca^{2+} cycling paces the heart. Incidentally, how do you explain that mice carrying the R4496C mutation, which strongly alters RyR2 function, have the same basal rate as WT animals [142]?

You wrote: "The discovery and functional importance of LCR's to normal automaticity of SANC has spawned a new breed of novel numerical models in which rate regulation is governed by intracellular Ca^{2+} cycling and its coupling to the surface membrane".

Ed, there are experimental data which contradict the assumption that rate regulation is governed by intracellular Ca^{2+} cycling, such as those concerning βAR rate modulation in the presence of ryanodine.

Disruption of Ca^{2+} cycling by ryanodine abolishes βAR-mediated rate acceleration, which led to the proposal of a direct role of Ca^{2+} transients in adrenergic rate modulation [85]. However, disruption of Ca^{2+} transients abolished βAR-induced cAMP synthesis, not the rate acceleration due to I_f activation. Indeed, perfusion of pacemaker cells with the membrane-permeable cAMP analogue CPT-cAMP led to rate acceleration (via the cAMP-I_f pathway) [99]. How could this happen if rate regulation were governed by Ca^{2+} cycling?

Based on this evidence, we suggested that inhibition of βAR rate acceleration could be caused by "perturbation of a Ca^{2+}-dependent element of the cAMP signaling cascade" such as a Ca^{2+}-dependent adenylate-cyclase, and that if a Ca^{2+}-dependent AC were "localized to the SAN then depletion of SR Ca^{2+} by ryanodine could result in inhibition of adenylate-cyclase function and disruption of βAR signaling" [99]. This prediction was indeed confirmed 4 years later by Terrar's group with the striking demonstration that the "neuronal" form of AC (AC1) is present in the guinea-pig SAN, but not in the ventricle [95]. This finding not only shed light on βAR rate modulation, it also provided the basis for a link between Ca^{2+} and I_f.

Conclusions

Ed, the evidence you discuss for the presence of a "tightly coupled system of SR Ca^{2+} cycling and surface membrane proteins" involving a complex array of cellular processes does not provide any proof that this mechanism ticks the heart. Ca^{2+} regulation is oscillatory because contraction is a repetitive phenomenon, and the fact that Ca^{2+} oscillates with rates

close to those of voltage is a clever self-arrangement selected by nature to allow entraining. A similar situation applies for example to circadian processes: all have a basic spontaneous rhythm of about 1 every 24 hours, but they need to be entrained by day/night cycling for perfect tuning.

I should like to return to the initial observation made in the Introductory Note, that is, funny channels are successful targets of specific heart-rate-reducing agents like ivabradine, whose efficiency in rate slowing (independently of how much this may affect morbidity-mortality of CAD patients) is out of doubt. This fact applies whatever the degree of complexity of processes involved in cell activity. Explaining the action of ivabradine with the assumption that I_f has no role in the control of heart rate is clearly unrealistic.

If on the other hand SR Ca^{2+} cycling is a real pacemaker, then we should expect that, sooner or later, a drug specifically reducing Ca^{2+} fluctuations rate will be developed, and perhaps even marketed, as a heart rate-reducing agent. Until then I believe that Ca^{2+} cycling should be regarded more as a pace-taker than a pacemaker mechanism.

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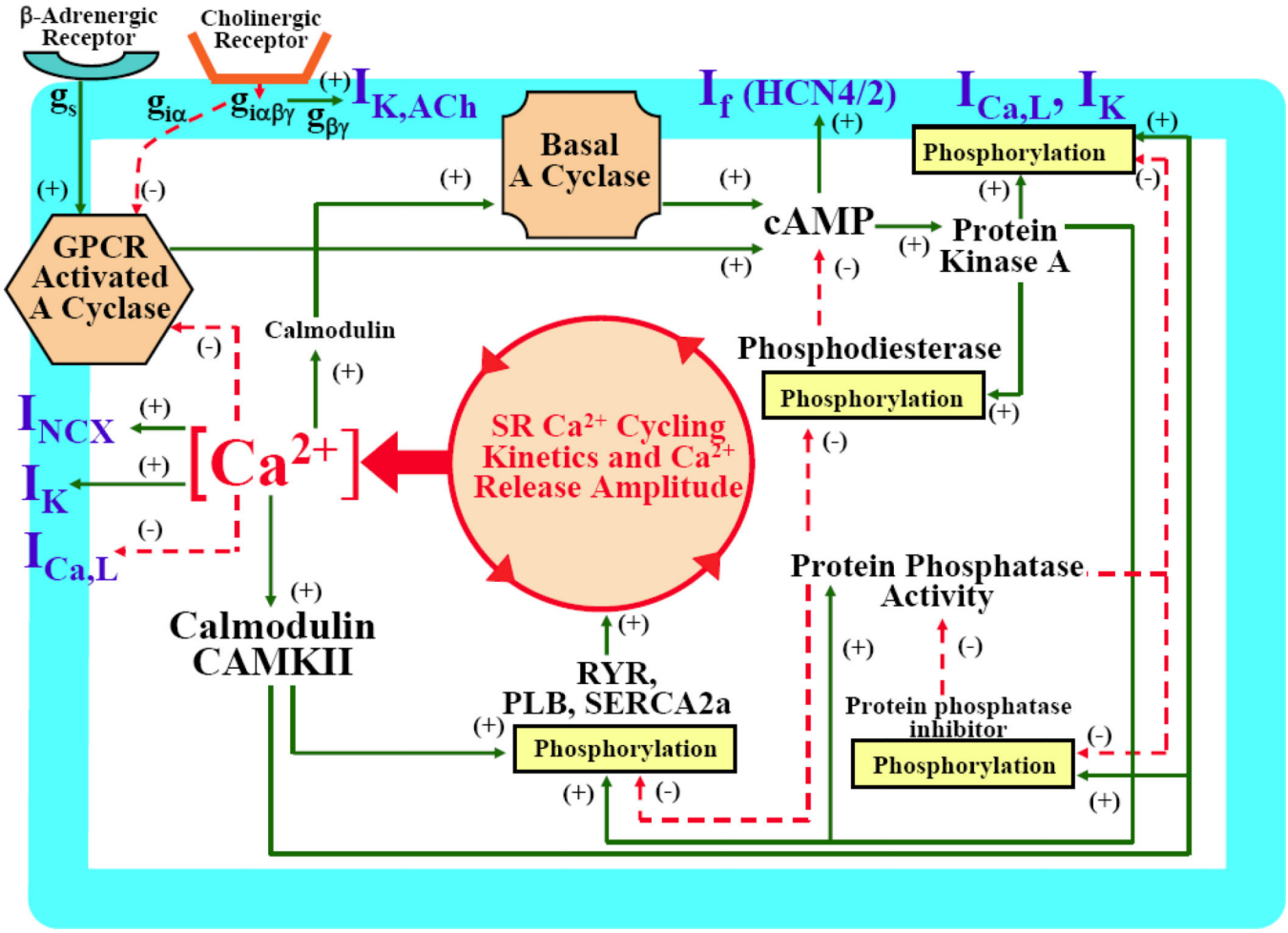

Fig. 1.

Schematic illustration of the interplay of Ca^{2+}, basal Ca^{2+} -activated AC, cAMP, PDE activity and PKA and CaMKII activity, cast in the context of sarcoplasmic reticulum Ca^{2+} cycling, L-type Ca^{2+} channels, and other ion channels. Spontaneous but rhythmic local submembrane Ca^{2+} releases during the later DD activate I_{NCX}, causing the DD to increase exponentially to achieve the threshold for I_{CaL} activation and the generation of the next AP. See text for further details. (adapted from [83]).

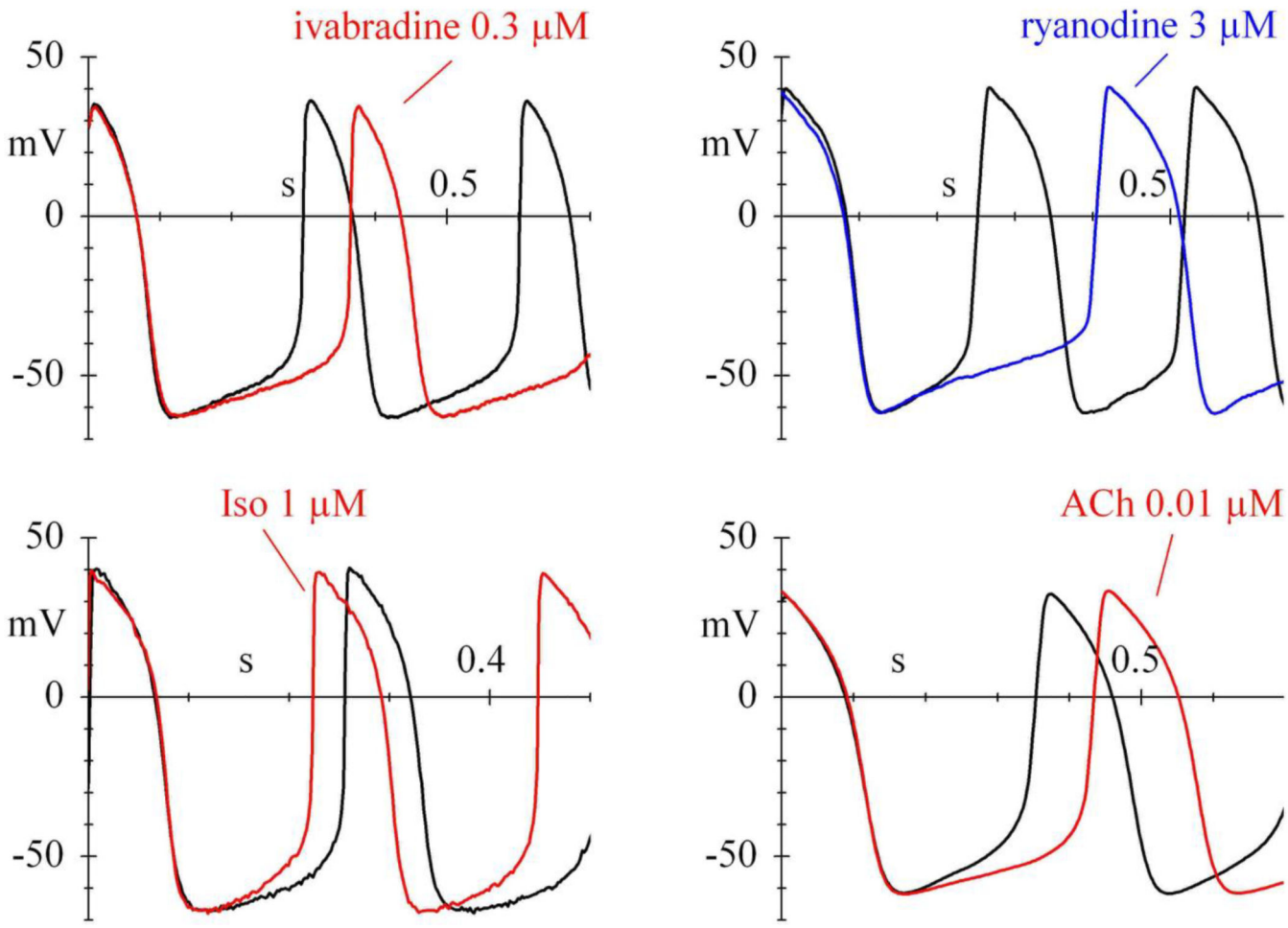

Fig. 2.

Action potentials recorded from rabbit SAN pacemaker cells in control conditions and in the presence of ivabradine 0.3 μM or ryanodine 3 μM (top) are compared with action potentials recorded before and during perfusion with the autonomic agonists Iso 1 μM or ACh 0.3 μM (bottom). Autonomic agonists modify rate by changing the slope of DD, including the early fraction, like ivabradine, while ryanodine affects mostly the late fraction of DD (partly adapted from [131]).