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Cardiac pacemaker cell failure with preserved I_f , I_{CaL} , and I_{Kr} : A lesson about pacemaker function learned from ischemia-induced bradycardia.

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Cardiac ischemia is an important global health problem. It leads to contraction failure, arrhythmias, and cardiac cell death. Sinus bradycardia characterized by a slow heart rate (<60 bpm) is a prominent ischemia-related arrhythmia directly caused by a deficiency of heart beat initiation within the sinoatrial node (SAN), due to a failure of the heart's primary pacemaker cells (SANC). The study presented by Yi-Mei Du and Richard Nathan in this issue of *Journal of Molecular and Cellular Cardiology* [1] deals with the ionic basis of ischemia-induced bradycardia in isolated SANC. The results of their study not only contribute to understanding of ischemia-induced bradycardia, but also help to delineate the fundamental mechanisms of cardiac pacemaker cell function.

Study design and hypothesis

In their studies, Du and Nathan simulated ischemia by superfusing SANC with a solution without glucose, at pH 6.6 that contained either 5.4 or 10 mM KCl. This lead, to a 13% or 43% reduction, respectively, in the spontaneous beating rate of freshly isolated SANC. This simulated ischemia is not true ischemia, e.g. there is no associated hypoxia or flow deficiency effects other than elevated $[K^+]$. Nonetheless simulated ischemia of this sort is often applied in studies like the present one. And, in their careful approach to the problem, the authors first demonstrated that their "ischemic" solutions produced bradycardia in the intact rabbit heart. Then, in isolated SANC they measured the effects of this simulated ischemia on membrane potential and ion currents under voltage clamp. Such an approach is not surprising, because it has been believed for almost half a century that the heart rhythm originates on the surface membrane of the cardiac pacemaker cells. This dogma stems from the Nobel prize triumph of the Hodgkin-Huxley neuron membrane excitability theory [2] and subsequent modification of this theory and extrapolation to the heart cells by Noble (1960) [3]. According to this theory, generation of spontaneous action potentials (APs) by cardiac pacemaker cells is portrayed as a membrane delimited process, i.e. by a pure interplay of time- and voltage- dependent ion currents. Pacemaker field researchers have focused mainly on the identification of multiple ion current components in pacemaker cells, and their respective roles in the spontaneous diastolic depolarization of these cells (DD, Fig. 1A) that brings the membrane to the excitation threshold (reviews [4,5]). Du and Nathan thus tested an hypothesis that bradycardia induced by their

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simulated ischemia is caused by attenuation of inward currents contributing to this most critical DD phase of the pacemaker AP.

Although Ca^{2+} was not measured by Du and Nathan in the present study of ischemic bradycardia, three of the inward currents assessed are directly related to Ca^{2+} cycling in the cells, i.e. T- and L- type Ca^{2+} currents and $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) current (I_{CaT} , I_{CaL} , I_{NCX} , respectively, Fig.1A, “Inward currents”). A plethora of recent data has emerged to conclusively show that intracellular Ca^{2+} dynamics, in tight cooperation with surface membrane proteins, are critical for the normal spontaneous firing of SANC (reviews [6,7]). Nathan’s group, in fact, was among the pioneers that discovered the major role of intracellular Ca^{2+} cycling in pacemaker function, showing that ryanodine, which interferes with Ca^{2+} release from the sarcoplasmic reticulum (SR), and BAPTA-AM, which chelates intracellular Ca^{2+} , significantly slowed the spontaneous beating rate of cardiac pacemaker cells [8–10]. The fourth inward current measured was the “funny” current (I_f) activated by membrane hyperpolarization, often referred to as “the” pacemaker current [11,12]. Finally, the authors completed their set of tested currents with an outward current, delayed rectifier K^+ current, specifically, its rapid component I_{Kr} , deactivation of which has a major contribution into the DD dynamics in rabbit SANC [13]. Thus, all studied currents are critically involved in either membrane-electrophysiology or intracellular Ca^{2+} dynamics during the DD. Other currents that have been thought to be involved in SANC pacemaker function [14] were not assessed by Du and Nathan.

Three major currents, I_f , I_{CaL} , and I_{Kr} do not fail when pacemaker function fails during ischemia

The results of the study showed that three major ion channels that contribute to the DD, activation of I_f and deactivation of I_{Kr} which both initiate the DD [12,13] and I_{CaL} , which becomes activated at the termination of the DD and produces the rapid upstroke of the AP [15] (Fig.1A), are not involved in the ischemia-induced rate reduction. I_{CaL} , in fact, increased, whereas I_{Kr} remained unchanged and I_f was either also unchanged or increased in 5.4 or 10 mM KCl, respectively. If these three prominent currents from the standpoint of membrane-delimited concept of the initiation of the heartbeat did not fail under ischemic-like conditions, then some other mechanism that is critical to normal pacemaker function must have failed.

I_{NCX} and I_{CaT} fail during ischemia

Du and Nathan have demonstrated that the pacemaker failure in the ischemia-like conditions is accompanied by a reduction of I_{CaT} and I_{NCX} , both known to contribute to the late DD [16–18]. This result suggests that one or both of these currents are critical to normal pacemaker function. In our opinion, the reduction in I_{CaT} is unlikely to be a major cause of ischemia-induced bradycardia on the basis of the following. The expression of I_{CaT} is strictly species dependent; current density of I_{CaT} in SAN is large in small animals and becomes smaller as body size increases (mouse > guinea-pig > rabbit > pig) [19]. Thus, it has been reasoned that in large mammals (e.g. pigs and humans), Ca^{2+} may be exclusively carried via I_{CaL} , and not I_{CaT} ; the lack of I_{CaT} in porcine SAN cells would be beneficial for slower pacemaker depolarization. Conversely, a high density of I_{CaT} in mouse SAN cells would promote pacemaker depolarization, thereby increasing heart rate [20]. Importantly, since I_{CaT} has never been detected in human heart tissue [19], clinically relevant ischemia-induced bradycardia in human SANC would not likely involve I_{CaT} . Thus, that I_{CaT} is so variable among species, and is even absent in some SANC, questions its fundamental importance to pacemaker function. Further, I_{CaT} has not been considered as a major component of the pacemaker mechanism of SANC, because a blockade of this current by a low concentration of Ni^{2+} results only in a minor change in the SANC beating rate (14–16% with 40–50 μM Ni^{2+} , respectively, [21,22]).

Du and Nathan did not evaluate the specific contribution of I_{CaT} to ischemic bradycardia. This might have been tested by supplementing their “ischemic” media with a similar low concentration of Ni^{2+} to block this current.

The key role of the NCX in pacemaker cell failure during ischemia: novel evidence for fundamental importance of NCX function

In our opinion, of the two inward currents that failed under ischemic conditions, the reduction in I_{NCX} is likely the major mechanism for ischemic bradycardia. Although Du and Nathan noted a significant effect of the “ischemic” solution on the DD slope, they assessed this as the average slope of the reduction of voltage throughout the entire DD time course. A closer inspection of their AP recordings, however, reveals that the DD is composed of an initial, linear part, followed later by a non-linear, exponentially rising part, which accelerates the change in membrane potential to achieve the AP threshold (See in Fig 1A “Linear DD” and “Nonlinear DD”, or in Fig.1B arrows b and c, respectively). This fine DD structure has been recently analyzed in great detail using a variety of experimental and numerical modeling approaches in rabbit SANC [18]. It was shown that the non-linear, exponentially rising, late DD part is formed by I_{NCX} (see in Fig.1A “ I_{NCX} ” and “Nonlinear DD” coincide). Notice, however, that while the ischemic solution changes the linear part of DD only slightly (if at all) (DD parts b and b' in Fig.1B), the major effect is clearly on the late, non-linear part (compare parts c and c' in Fig.1B). So, the non-linear DD part, in fact, becomes “missing” in the ischemic solution (DD part b'-c' is almost straight in Fig.1B). This major divergence of the DD from its normal shape thus ought to be produced by the reduction of I_{NCX} found by Du and Nathan in the ischemic solution. The corollary of the demonstration that I_{NCX} is markedly reduced by simulated ischemia is that I_{NCX} must be also critical to normal pacemaker function, thus providing a new additional piece of evidence for the fundamental importance of the NCX for SANC function suggested previously by different research groups [7,17,23].

How cell Ca^{2+} cycling links to NCX function and why it is critical for pacemaker function

So, what determinants of I_{NCX} render its function to be crucial to normal pacemaker cell beating? In contrast to other currents tested in the study, I_{NCX} is carried not by an ordinary ion channel, but an ion transporter, NCX. It is activated by an increase in intracellular $[Ca^{2+}]$, and once activated this way, it works in so called “forward mode” exchanging 1 Ca^{2+} for 3 Na^+ thus generating an inward current [24] at diastolic potentials. A great benefit to have NCX in the plasma membrane is that it can transform and therefore couple “chemical” signals of Ca^{2+} cycling within pacemaker cells into “electrical” signals of membrane depolarization followed by an excitation.

Ca^{2+} cycling in cardiac cells is characterized by two major types of Ca^{2+} releases from SR: global, AP-synchronized Ca^{2+} transients and spontaneous, local Ca^{2+} releases (LCRs) (recent reviews [6,7]). The essence of Ca^{2+} cycling in normal ventricular muscle cells is the Ca^{2+} transient, which is initiated and entrained by externally delivered APs, and serves as a high gain signal amplifier within Ca^{2+} -induced- Ca^{2+} -release (CICR) paradigm. Since NCX function is both Ca^{2+} - and voltage- dependent, at the peak of the AP it operates in the “reverse mode”, transferring more Ca^{2+} into cells; then, as the membrane potential repolarizes and crosses the reversal potential of the NCX, it operates in the “forward mode” helping to remove the cytosolic Ca^{2+} , thus allowing the muscle to relax. Spontaneous LCRs usually do not occur between beats in ventricular myocytes during their normal operation. Under excessive SR Ca^{2+} loading conditions, however, diastolic LCRs initiate Ca^{2+} waves at the diastolic membrane potentials

and activate inward NCX current that has been linked to the diastolic membrane depolarizations that have been implicated in ventricular arrhythmias (review [25]).

The normal Ca^{2+} cycling in SANC is different from that of ventricular cells. During each beat, in the absence of Ca^{2+} overload, SANC manifest both an AP-induced Ca^{2+} transient and, importantly, spontaneous Ca^{2+} releases, i.e. LCRs in the form of Ca^{2+} sparks and small Ca^{2+} waves during the late DD phase [17,26] (Fig. 1A, “ Ca^{2+} dynamics”). A recent discovery explained this fundamental difference of Ca^{2+} cycling in these two cell types. It turned out that in contrast to rabbit ventricular myocytes, Ca^{2+} cycling proteins, e.g. phospholamban, ryanodine receptors, and likely L-type Ca^{2+} channels in rabbit SANC, are highly phosphorylated in the basal state [27] (for L-type Ca^{2+} channels see [28]). PKA-dependent phosphorylation of these proteins presents the SR with an increased Ca^{2+} to be pumped (Ca^{2+} influx via I_{CaL}), speeds up the SR Ca^{2+} filling rate (phospholamban), and alters the Ca^{2+} release threshold (ryanodine receptors). The net effects of this phosphorylation create conditions that are required for LCR spontaneous activation during the DD. The LCR activation in rabbit SANC is indeed truly spontaneous as it does not depend on membrane depolarization (but see [29] for cat latent atrial pacemaker cells), and can be observed under voltage clamp or in permeabilized SANC [27,30]. Importantly, it is the spontaneous emergence of LCRs during the late DD that activates I_{NCX} and thus changes the DD dynamics from a linear to a nonlinear, exponentially rising function, culminating in I_{CaL} activation and membrane excitation (Fig. 1A) [17,18].

Ca^{2+} cycling within cardiac pacemaker cells is a “Clock”

Spontaneous diastolic LCRs are manifestation of an internal “Clock” that generates rhythmic intracellular Ca^{2+} signals (Fig. 1A, see Ca^{2+} clock “ticks”, red) that are transformed by NCX to DD acceleration to prompt rhythmic AP’s. The speed at which the Ca^{2+} clock runs is assessed by the LCR period, defined as the time from the AP-induced Ca^{2+} transient early in the cycle (Fig. 1A, Clock “reset”, blue) to LCR emergence during the subsequent DD (Clock “tick”). This Ca^{2+} clock’s period is slightly shorter than the cycle length (Fig. 1A) and approximately coincides with the period of the intrinsic Ca^{2+} oscillations under voltage clamp [27,30]. The speed at which Ca^{2+} clock ticks is variable, matching the chronotropic demand for a given condition, and is governed by the SR Ca^{2+} loading and Ca^{2+} release characteristics, which in turn, are governed by the degree of phosphorylation of its aforementioned Ca^{2+} cycling proteins. This phosphorylation-graded variability of the Ca^{2+} clock is thus the essence of basal rate regulation and regulation of chronotropic reserve of SANC by neuromodulators [27].

While the Ca^{2+} clock keeps the time of the SANC pacemaker, it is tightly integrated with the plasma membrane proteins (including both electrogenic process and Ca^{2+} transport) resulting in an extremely robust pacemaker operation [6,7]: Rhythmic LCRs excite the cell surface membrane via activation of inward NCX current during the late DD phase, but AP-induced Ca^{2+} influx in turn, depletes SR Ca^{2+} content (Fig. 1A, Ca^{2+} Clock “reset”) via ClCR ; and it also balances the NCX-related Ca^{2+} efflux (“refueling” Ca^{2+} Clock).

It is important to note, although membrane Ca^{2+} influx and SR Ca^{2+} cycling are integrated, a failure to generate the critical LCR/NCX signals leads to pacemaker failure, even when Ca^{2+} influx via I_{CaL} is normal or even enhanced (as in this study by Du and Nathan). A secondary role of I_{CaL} in SANC pacemaker function has also been demonstrated by observations that β -adrenergic receptor (βAR) stimulation still enhances I_{CaL} under ryanodine, but the chronotropic effect of this stimulation is markedly blunted (both in vitro and in vivo) [22,27].

The study by Du and Nathan provides additional, important evidence for the above idea of Ca^{2+} -integrated pacemaking, i.e., that normal, robust pacemaker function requires the activation of LCRs and I_{NCX} during the late DD, that crucial time when I_f has faded, but

I_{CaL} is not yet activated (Fig.1A). In other words, whereas both I_f and I_{Kr} determine the maximum diastolic potential and initiate the early linear part of the DD [12,13], this later critical I_{NCX} prompt is required to steadily and timely bring the membrane potential to the AP threshold. Indeed, while the increase in I_{CaL} found under ischemic-like conditions results in a lower take-off potential, as seen in Fig.1B, this does not compensate for the absence of an exponentially rising, late DD phase resulted from the deficiency of NCX function. That the impact of a greatly increased I_f under ischemic-like conditions in the study by Du and Nathan fails to drive membrane potential towards the lower take-off potential, similarly questions a major role of this current in the pacemaker function in these cells. A minor role of I_f in cardiac pacemaker mechanism has been previously suggested based on a variety of considerations, including its activation kinetics, voltage-dependency, a minor change of the beating rate after its blockade, and a minor role in pacemaker rate regulation [22,31,32].

Du and Nathan also observed the blocked APs under ischemic-like conditions. Such AP failure also occurs when Ca^{2+} and membrane interaction via NCX is blocked in Na^+ -free bath solution or when normal Ca^{2+} cycling is compromised by ryanodine or protein kinase-A (PKA) inhibition. In such instances, the DD does begin, but then decelerates and ultimately fails to ignite an excitation [17,27,32,33]. This means, generally, that whatever the factor (ischemia-like conditions, Na^+ -free bath solution, PKI, ryanodine, etc.) that interferes with NCX or Ca^{2+} cycling, it has the same major consequences: depending on dose and time, beating rate is greatly slowed, become irregular, and may eventually cease (Fig.1C). This is in distinct contrast to relatively minor effects of blockade of ion channels such as I_f and I_{CaT} (Fig.1C).

What causes the decrease of I_{NCX} in ischemia?

Previous experiments showed that interventions that inhibit LCRs markedly reduce the nonlinear DD component and markedly reduce or abolish spontaneous beating; on the other hand, selectively blocking NCX, with LCRs remaining intact, also reduce the late DD and markedly reduce or abolish spontaneous beating [17,18](Fig.1C). Accordingly, the ischemia-induced reduction of I_{NCX} could be due to altered Ca^{2+} cycling described above and/or a direct effect on NCX function. Evidence that ischemia influences Ca^{2+} cycling and I_{NCX} has accumulated since the late 1970s-early 1980s, when Ca^{2+} cycling by SR came into focus as the major mechanism of cardiac cell function (review [25]). Under steady-state conditions the direct effect of acidosis on the SR in skinned cardiac cells [34,35] and in SR vesicles [36] is to decrease Ca^{2+} uptake and thus subsequent release. Studies in intact hearts showed that ischemia reduced spontaneous Ca^{2+} release (indexed as scattered light intensity fluctuations generated by Ca^{2+} -dependent myofilament motion) to zero within approximately 30 min [37, 38]. Recent studies in toad pacemaker cells by Ju and Allen 2003 [39] showed that metabolic inhibition, used to simulate ischemia, decreases Ca^{2+} release from the SR, mainly by reducing SR Ca^{2+} content due to decreased Ca^{2+} uptake by the SR pump.

Du and Nathan found that their “ischemic” solution reduced inward current tails that were sensitive to high concentrations of Ni^{2+} (presumably I_{NCX}), even in the presence of ryanodine, when SR Ca^{2+} ought to be markedly reduced. Also, after exposing freely firing SANC to ryanodine for unspecified time, the “ischemic” solution still slowed their firing rate. Based on these finding, Du and Nathan reasoned that this could be due to a *direct* effect of simulated ischemia on NCX; in other words, a reduction in LCRs is not sufficient to explain full effect of simulated ischemia to reduce I_{NCX} . While a direct effect of acidosis [40], presumably due to a change in electrogenicity of the NCX protein [41], seems to be a plausible explanation, the latter interpretation of the further reduction of the beating rate by the “ischemic” solution in the presence of ryanodine needs careful consideration. Based on a recently reported summary of available data of ryanodine effects in SANC (Table 1 in [32]), ryanodine concentrations ranging from 1 to 30 μM produced rate reductions from 19% to 100%. Ryanodine, in a

concentration of 40 μM employed by Du and Nathan, is, in fact, a relatively high concentration of the drug. It is puzzling to us why this resulted only in 12% slowing of the beating rate in their experiments. The ryanodine effect on Ca^{2+} cycling has a complex dynamics: firstly, it increases LCRs from the SR and this is accompanied by an increase in the beating rate; this is followed by SR Ca^{2+} depletion, cessation of LCRs, and by a slowing of the beating rate, disturbances in rhythm, and ultimately, failure of spontaneous beating. So, the precise times at which the “ischemic” solution is applied and at which the measurements are taken may yield greatly different results. More specifically, after a small initial rate reduction (such as 12%), the subsequent effect of “ischemic” solution might, in fact, overlap with continuing dynamics of the ryanodine effect, thus complicating interpretation of the data. Direct measurement of Ca^{2+} , particularly LCRs, are required in this regard.

Summary and conclusions

The study by Du and Nathan in this issue of *Journal of Molecular and Cellular Cardiology* [1] suggests that simulated ischemia-induced bradycardia in rabbit SANC is caused, at least in part, by a failure (i.e. reduction) of two ion currents I_{CaT} and I_{NCX} . However, the extent to which each of these reductions contributes to bradycardia was not determined. Since previous studies demonstrated a minor role of I_{CaT} in normal pacemaker function of rabbit SANC, the reduction in I_{NCX} rather than that in I_{CaT} is likely the major mechanism in the simulated ischemia-induced bradycardia. The finding of that other major currents I_{CaL} , I_{f} , and I_{Kr} , contributing to the diastolic depolarization, do not fail under ischemic-like conditions, whereas NCX does, suggests that the latter is critical to the normal pacemaker function. This idea is in line with direct, recent demonstrations in other studies of rabbit SANC [17,18] of the key role of NCX in normal pacemaker function. Furthermore, because NCX is activated by intracellular Ca^{2+} , a broad interpretation of the results of this study is that pacemaker failure during simulated ischemia is not caused solely by ion channel failure, but also by altered SR Ca^{2+} cycling and/or a compromised intracellular Ca^{2+} integration with the membrane excitation due to the deficiency of NCX function. This interpretation supports the novel hypothesis [7] that normal cardiac pacemaker function requires functional integration of internal Ca^{2+} cycling with membrane-delimited electrogenic proteins. According to this Ca^{2+} integrated pacemaker concept, spontaneous local Ca^{2+} releases emerging during the late DD activate an ensemble of local inward NCX currents; this accelerates DD and ignites I_{CaL} to generate an AP (Fig. 1A). This DD acceleration is clearly demonstrated in the Du and Nathan’s recordings of spontaneous APs, and it is the late, non-linear DD part (associated with normal SANC operation) does fail under ischemia-like conditions (Fig.1B). While previous experiments demonstrated that ischemia interferes with the SR Ca^{2+} cycling in different cardiac cell types, unfortunately Ca^{2+} measurements were not performed in this study. A direct evaluation of local spontaneous Ca^{2+} releases during the late DD of SANC in future studies of ischemia-induced bradycardia merits consideration.

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Figure 1.

A: A schematic illustration of the fine structure of the diastolic depolarization (DD) of spontaneous action potential in rabbit SANC shown together with inward ion currents assessed in the study by Du and Nathan [1] and related Ca^{2+} signals (see text for detail). The nonlinear, exponentially rising, late DD part is shown as a gray area between membrane potential curve and an extrapolation of the initial, linear DD part. The action potential shapes are taken from the two first action potentials presented in Fig.1A of the study by Du and Nathan [1]. B: The "ischemic" solution mainly reduces the nonlinear, late DD part (c), rather than the early part (b) of the DD. Shown is a copy of the Figure 1G from [1]. C: Spontaneous beating of rabbit SANC critically depends upon Ca^{2+} related mechanisms and protein phosphorylation. Bars

show a decrease in the beating rate (% control) induced by different drugs that affect Ca^{2+} cycling [ryanodine (Ry), BAPTA-AM], NCX (Li^+ substitution for Na^+), protein phosphorylation (PKI, H-89, MDL), or ion currents: I_f (Cs^+) or T-type Ca^{2+} current (Ni^+). From [6]. PKI and H-89 are protein kinase A inhibitors and MDL is an adenylyl cyclase inhibitor.