Journal of Molecular and Cellular Cardiology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

New evidence for coupled clock regulation of the normal automaticity of sinoatrial nodal pacemaker cells: Bradycardic effects of ivabradine are linked to suppression of intracellular Ca^{2+} cycling

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ARTICLE INFO

Article history:
Received 7 December 2012
Received in revised form 7 April 2013
Accepted 29 April 2013
Available online xxxx

Keywords:
Sinoatrial nodal pacemaker cells
Ca^{2+} cycling
Ion channels
Physiology
Sarcoplasmic reticulum

ABSTRACT

Beneficial clinical bradycardic effects of ivabradine (IVA) have been interpreted solely on the basis of I_f inhibition, because IVA specifically inhibits I_f in sinoatrial nodal pacemaker cells (SANC). However, it has been recently hypothesized that SANC normal automaticity is regulated by crosstalk between an "M clock," the ensemble of surface membrane ion channels, and a "Ca^{2+} clock," the sarcoplasmic reticulum (SR). We tested the hypothesis that crosstalk between the two clocks regulates SANC automaticity, and that indirect suppression of the Ca^{2+} clock further contributes to IVA-induced bradycardia. IVA (3 μM) not only reduced I_f amplitude by 45 ± 6% in isolated rabbit SANC, but the IVA-induced slowing of the action potential (AP) firing rate was accompanied by reduced SR Ca^{2+} load, slowed intracellular Ca^{2+} cycling kinetics, and prolonged the period of spontaneous local Ca^{2+} releases (LCRs) occurring during diastolic depolarization. Direct and specific inhibition of SERCA2 by cyclopiazonic acid (CPA) had effects similar to IVA on LCR period and AP cycle length. Specifically, the LCR period and AP cycle length shift toward longer times almost equally by either direct perturbations of the M clock (IVA) or the Ca^{2+} clock (CPA), indicating that the LCR period reports the crosstalk between the clocks. Our numerical model simulations predict that entrainment between the two clocks that involves a reduction in I_{NCX} during diastolic depolarization is required to explain the experimentally AP firing rate reduction by IVA. In summary, our study provides new evidence that a coupled-clock system regulates normal cardiac pacemaker cell automaticity. Thus, IVA-induced bradycardia includes a suppression of both clocks within this system.

1. Introduction

Results of recent clinical trials (BEAUTIFUL, SHIFT, VIVIFY, etc.) indicate that a reduction in heart rate by ivabradine (IVA) was anti-anginal and anti-ischemic (for review cf. [1]). Moreover, IVA has been demonstrated to reduce diastolic dysfunction and cardiac fibrosis [2] and to increase exercise capacity [3]. In a model of myocardial ischemia/reperfusion, IVA improved the regional myocardial blood flow and function and reduced the infract size [4]. In isolated sinoatrial nodal pacemaker cells (SANC) under voltage clamp and buffered Ca^{2+} conditions (i.e., presence of EGTA in the patch pipette), IVA, in low doses, specifically inhibits I_f of SANC but not other membrane currents [5,6]. The beneficial clinical bradycardic effects of IVA have been interpreted solely on the basis of I_f inhibition [1–4,7].

However, accruing evidence has been interpreted to indicate that normal automaticity of the SANC is regulated by integrated functions within a system of two clock-like oscillators [8–10]: the sarcoplasmic reticulum (SR), acting as a "Ca^{2+} clock," rhythmically discharges diastolic local Ca^{2+} releases (LCRs) beneath the cell surface membrane; LCRs activate an inward current (likely that of the Na^+/Ca^{2+} exchanger) that prompts a surface membrane or "M clock," the ensemble of sarcolemmal electrogenic molecules, to effect an action potential (AP) (Fig. 1). Periodicity of Ca^{2+} clock-generated LCRs is regulated by the SR Ca^{2+} pumping, which depends not only on SR proteins (phospholamban (PLB) and ryanodine receptors), and their phosphorylation status, but also on function and phosphorylation status of M clock proteins, e.g., L-type Ca^{2+} channels that regulate cell Ca^{2+} available for SR pumping. According to the coupled-clock hypothesis [11], the M and Ca^{2+} clocks should crosstalk. Possibilities for crosstalk and feedback via changes in SR Ca^{2+} cycling include Ca^{2+}-dependent electrogenic processes (such as Na^+/Ca^{2+} exchange) and voltage-dependent Ca^{2+} fluxes (such as via L-type Ca^{2+} channels). Thus, a change in AP firing rate in response to any disturbance signal that perturbs either clock

Abbreviations: AC, Adenylyl-cyclases; AP, Action potential; CPA, Cyclopiazonic acid; IVA, Ivabradine; LCR, Local Ca^{2+} release; M, Membrane; MDP, Maximum diastolic depolarization; PKA, Protein kinase A; PLB, Phospholamban; SANC, Sinoatrial-node cells; SR, Sarcoplasmic reticulum; T-50_c, 50% decay time of intracellular Ca^{2+}; T-90_c, 90% decay time of intracellular Ca^{2+}.

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0022-2828/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.yjmcc.2013.04.026

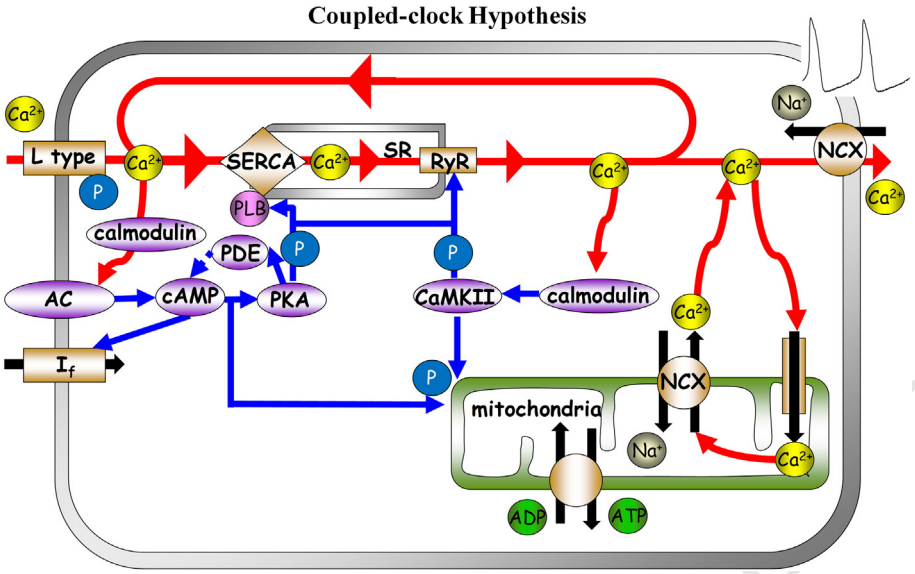

Fig. 1. Schematic illustrations of the coupled clock system. The interplay of Ca^{2+}-calmodulin adenylyl-cyclases (AC), PDE activity and cAMP-mediated, protein kinase A (PKA)-dependent and Ca^{2+}/calmodulin protein kinase II (CaMKII) signaling to the cardiac pacmaker sarcoplasmic reticulum Ca^{2+} cycling proteins, surface membrane ion channels and mitochondria.

entrains the function of the other clock. This is followed by a feedback from the entrained clock to the originally perturbed clock. Such feedback amplifies the response of the initially perturbed clock to the original signal, ensuring a robust response to the initial disturbance signal. Therefore, the steady-state AP cycle length change embodies contributions of both clocks, as in other coupled oscillatory systems throughout nature [12]. Because both M and Ca^{2+} clock molecules regulate cellular Ca^{2+} homeostasis, and resultant LCR characteristics, the coupled-clock hypothesis also predicts that in response to any chronotropic perturbation, the net changes in the steady-state LCR period are a reflection of integrated clock function.

Although the coupled-clock hypothesis is conceptually attractive, direct experimental evidence to support crosstalk between the two clocks is lacking, and the idea that normal automaticity of SANC is regulated by a coupled-clock system remains controversial. We sought to provide evidence for crosstalk between the two clocks by determining whether the effect of IVA, at a concentration that specifically inhibits I_f of the M clock to reduce the AP firing rate, is accompanied by effects on the intracellular Ca^{2+} cycling, i.e. on the "Ca^{2+} clock."

We further approached this clock crosstalk by determining the ability for observed changes in Ca^{2+} cycling to feed back to the M clock, by specific perturbation of the Ca^{2+} clock (using pharmacological inhibition of SR Ca^{2+} pump). We also further explore the detailed mechanisms of the crosstalk using numerical model simulations that embrace AP, I_f, $I_{Ca,L}$ and Ca^2-dependent phosphorylation effect on SR Ca^{2+} loading and release. Our discovery of the Ca^{2+} component in the bradycardic effects of IVA in cardiac pacemaker cells is clinically relevant, because this indicates that targeting the coupled-clock system within pacemaker cells, rather than a single effector molecule, such as funny channel, is likely to be a more general and effective strategy to normalize heart rate in patients requiring chronotropic therapy. Moreover, our results reveal the presence of powerful feed-forward and feed-back coupling mechanisms in SANC, providing new evidence that coupled-clock functions confer robustness to normal pacemaker cell automaticity.

2. Methods

To test the hypothesis that the AP cycle length is regulated by crosstalk between surface membrane ion channels and intracellular Ca^{2+} cycling, we superfused single, isolated rabbit SANC with IVA, a direct and specific blocker of I_f, or cyclopiazonic acid (CPA) a direct and specific inhibitor of Ca^{2+} pumping by SERCA2. We measured AP cycle length, cytosolic Ca^{2+}, SR Ca^{2+} load and LCR characteristics during diastolic depolarization in spontaneously beating SANC, and also LCR characteristics in permeabilized SANC. In addition, we measured I_f and $I_{Ca,L}$ in voltage-clamped SANC. We determined whether simulations of our mathematical model could reproduce the experimental results, i.e., whether the change in AP cycle length in response to IVA requires crosstalk between membrane ionic currents and SR Ca^{2+} fluxes. A detailed expanded description of experimental and numerical methods is available in the Online Data Supplement. The experiment protocols have been approved by the Animal Care and Use Committee of the National Institutes of Health (protocol #034LCS2013).

3. Results

3.1. IVA blocks I_f in SANC

We first verified whether the previously determined effects of 3 μM IVA to inhibit I_f via whole cell voltage clamp in rabbit SANC [6] are reproducible in single, isolated rabbit SANC studied under our conditions. A representative example of the effect of IVA (3 μM) on I_f over a range of membrane potentials is shown in Fig. 2A, and the average I_f characteristics in the presence or absence of IVA are summarized in Table S3. The average effects of IVA (n = 9) on the I–V relationships of peak I_f are shown in Fig. 2B. At the maximum diastolic depolarization (MDP) during spontaneous AP firing (~−59 mV; Table S4), IVA decreased peak I_f, on average, by 45 ± 6% control (Fig. 2B inset). I_f activation (Fig. 2C) was calculated from the peak tail current 5 min after IVA application (i.e., during steady state I_f blockade). On average, IVA decreased peak I_f without significantly shifting its voltage-dependent activation (Fig. 2D, Table S3). Note that in spontaneously beating SANC, unlike in the case of voltage clamp, a long time between spontaneous APs might increase I_f activation that may contradict the effect of IVA to reduce I_f amplitude at MDP.

3.2. Effects of IVA on the SANC spontaneous AP firing rate, intracellular Ca^{2+} cycling and SR Ca^{2+} load

To determine the effect of IVA on AP firing rate and AP parameters, we superfused single SANC with IVA for 10 min. Representative