Journal of Molecular and Cellular Cardiology 66 (2014) 106–115

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Sarcoplasmic reticulum Ca^{2+} cycling protein phosphorylation in a physiologic Ca^{2+} milieu unleashes a high-power, rhythmic Ca^{2+} clock in ventricular myocytes: Relevance to arrhythmias and bio-pacemaker design

Syevda Sirenko [a], Victor A. Maltsev [a], Larissa A. Maltseva [a], Dongmei Yang [a], Yevgeniya Lukyanenko [a], Tatiana M. Vinogradova [a], Larry R. Jones [b], Edward G. Lakatta [a,*]

[a] Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA
[b] Department of Medicine, Krannert Institute of Cardiology, Indianapolis, IN, USA

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form 7 November 2013
Accepted 14 November 2013
Available online 22 November 2013

Keywords:
Cardiac ventricular myocytes
Calcium clock
Calcium cycling
Protein phosphorylation
Spontaneous local calcium releases

ABSTRACT

Basal phosphorylation of sarcoplasmic reticulum (SR) Ca^{2+} proteins is high in sinoatrial nodal cells (SANC), which generate partially synchronized, spontaneous, rhythmic, diastolic local Ca^{2+} releases (LCRs), but low in ventricular myocytes (VM), which exhibit rare diastolic, stochastic SR-generated Ca^{2+} sparks. We tested the hypothesis that in a physiologic Ca^{2+} milieu, and independent of increased Ca^{2+} influx, an increase in basal phosphorylation of SR Ca^{2+} cycling proteins will convert stochastic Ca^{2+} sparks into periodic, high-power Ca^{2+} signals of the type that drives SANC normal automaticity. We measured phosphorylation of SR-associated proteins, phospholamban (PLB) and ryanodine receptors (RyR), and spontaneous local Ca^{2+} release characteristics (LCR) in permeabilized single, rabbit VM in physiologic [Ca^{2+}], prior to and during inhibition of protein phosphatase (PP) and phosphodiesterase (PDE), or addition of exogenous cAMP, or in the presence of an antibody (2D12), that specifically inhibits binding of the PLB to SERCA-2. In the absence of the aforementioned perturbations, VM could only generate stochastic local Ca^{2+} releases of low power and low amplitude, as assessed by confocal Ca^{2+} imaging and spectral analysis. When the kinetics of Ca^{2+} pumping into the SR were increased by an increase in PLB phosphorylation (via PDE and PP inhibition or addition of cAMP) or by 2D12, self-organized, "clock-like" local Ca^{2+} releases, partially synchronized in space and time (Ca^{2+} wavelets), emerged, and the ensemble of these rhythmic local Ca^{2+} wavelets generated a periodic high-amplitude Ca^{2+} signal. Thus, a Ca^{2+} clock is not specific to pacemaker cells, but can also be unleashed in VM when SR Ca^{2+} cycling increases and spontaneous local Ca^{2+} release becomes partially synchronized. This unleashed Ca^{2+} clock that emerges in a physiological Ca^{2+} milieu in VM has two faces, however: it can provoke ventricular arrhythmias; or if harnessed, can be an important feature of novel bio-pacemaker designs.

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1. Introduction

Spontaneous, rare, stochastic local diastolic Ca^{2+} releases ("Ca^{2+} sparks") [1] that occur in basal-state cardiac ventricular myocytes (VM) provide an important SR Ca^{2+} leak pathway [2]. β-adrenergic receptor stimulation (β-AR_S) of VM organizes those local diastolic Ca^{2+} releases into partially synchronized spontaneous, periodic diastolic Ca^{2+} signals (Ca^{2+} waves) that, unlike "Ca^{2+} sparks", can be of sufficient amplitude to generate abnormal spontaneous diastolic afterdepolarizations that can initiate spontaneous abnormal action potentials (AP_S) [3]. During β-AR_S, two distinct, but related, phosphorylation-dependent events occur: (i) an increase in Ca^{2+} influx into the cell, and (ii) increased Ca^{2+} pumping rate into and release from SR. An increase in intracellular Ca^{2+}, due to an increase in Ca^{2+} influx effected by a β-AR_S-induced increase in phosphorylation of L-type Ca^{2+} channel subunits is thought to be the major mechanism involved in organization of local, stochastic Ca^{2+} signals into spontaneous, roughly periodic Ca^{2+} waves [4]. One viewpoint, however, is that, although β-AR_S initially increases Ca^{2+} influx, the steady-state cell Ca^{2+} load during β-AR_S does not increase (vs. that in the basal state), because Ca^{2+} efflux from the cell increases to match influx [5]. Sarcoplasmic reticulum (SR) Ca^{2+} cycling proteins, e.g. phospholamban (PLB) and ryanodine receptors (RyRs) also become phosphorylated during β-AR_S, and an increase

Abbreviations: AP, action potential; β-ARs, β-adrenergic receptor stimulation; Ca^{2+}, calcium; IBMX, isobutyl-1-methylxanthine; LCR, local Ca^{2+} releases; PDE, phosphodiesterase; PKA, protein kinase A; PLB, phospholamban; PP, protein phosphatases; RyRs, ryanodine receptors; SANC, sinoatrial node cells; SERCA-2, SR Ca^{2+} pump; SR, sarcoplasmic reticulum; VM, ventricular myocytes.

* Corresponding at: Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, NIH, 251 Bayview Blvd., Baltimore, Maryland 21224, USA. Tel.: +1 410 558 8202.
E-mail address: LakattaE@mail.nih.gov (E.G. Lakatta).

0022-2828/$ – see front matter. Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.yjmcc.2013.11.011

in the phosphorylation state is associated with enhanced Ca^{2+} pumping into SR, and to changes in spontaneous activation of RyRs. A role for enhanced SR Ca^{2+} cycling in the organization of partially synchronized, roughly periodic spontaneous diastolic SR Ca^{2+} releases in VM, in the absence of Ca^{2+} overload, however, has not been directly demonstrated.

A clue that increased SR Ca^{2+} cycling in the absence of Ca^{2+} overload can indeed generate roughly periodic spontaneous local Ca^{2+} releases (referred as "LCRs"), however, has emerged from recent studies in sinoatrial nodal pacemaker cells (SANC), in which basal levels of phosphorylation of Ca^{2+} cycling proteins are well above those in basal VM in a physiologic Ca^{2+} milieu [6]. These studies in SANC, in which the surface membrane had been permeabilized, clearly demonstrated that an enhanced rate of SR Ca^{2+} cycling effected by increased basal phosphorylation of SR Ca^{2+} cycling proteins enables inherently stochastic, sub-sarcolemmal LCRs via RyRs to become organized into roughly periodic Ca^{2+} signals (Ca^{2+} wavelets), even when the ambient steady [Ca^{2+}] is buffered constantly at physiologic levels [6,7]. LCRs are Ca^{2+} wavelets, i.e. larger and more organized than Ca^{2+} sparks, but, unlike Ca^{2+} waves, propagate only locally for relatively short distances (3 to 7 μm). Since SR generated LCRs are roughly periodic, the SR in SANC has been dubbed a "Ca^{2+} clock" [8].

In SANC with intact sarcolemma, spontaneous, periodic Ca^{2+} wavelets during diastole generated by the SR Ca^{2+} clock are of sufficient amplitude to effect local membrane depolarization (via activation of Na^+/Ca^{2+} exchanger) that are critically linked to the occurrence of spontaneous, rhythmic APs, i.e. normal automaticity of the cardiac impulse [8]. Since high basal levels of Ca^{2+} cycling protein phosphorylation in SANC organize stochastic Ca^{2+} releases into local wavelet-like rhythmic LCRs, i.e. Ca^{2+} clock, we hypothesized that suppression of basal SR Ca^{2+} cycling in VM linked to a suppression of basal phosphorylation of SR Ca^{2+} cycling, prevents the emergence of periodic, organized LCRs (i.e. prevents the emergence of a Ca^{2+} clock in VM); instead only stochastic, low-amplitude "Ca^{2+} sparks" occur. Specifically, we hypothesized that even in a physiologic Ca^{2+} milieu, when the basal SR Ca^{2+} cycling rate increases, e.g. either in response to an increase in SR Ca^{2+} protein phosphorylation in VM when protein phosphatase (PP) and phosphodiesterase (PDE) activities are inhibited, or when PLB-SERCA interaction is inhibited by a specific monoclonal antibody, spontaneous stochastic sparks will self-organize into synchronized, periodic LCRs, i.e., a "Ca^{2+} clock" will emerge in VM.

2. Methods

Spontaneous local Ca^{2+} release characteristics (LCR), the phosphorylation status of SR-associated proteins, PLB and RyRs in permeabilized rabbit VM bathed in 100 nM free [Ca^{2+}], and cytosolic Ca^{2+} signal in electrically stimulated rabbit VM with intact sarcolemma were measured. Shortly, intact VM were permeabilized with 0.01% saponin. After washing out saponin, solution was exchanged to the recording solution that contained 0.03 mM fluo-4 pentapotassium salt, 0.114 mM $CaCl_2$ (free [Ca^{2+}] ~ 100 nM), 100 mM $C_4H_6NO_4K$ (DL-aspartic acid potassium salt), 25 mM KCl, 10 mM NaCl, 3 mM MgATP, 0.81 mM $MgCl_2$, 20 mM Hepes, 0.5 mM EGTA, 10 mM phosphocreatine, and creatine phosphokinase (5 U/ml), pH 7.2 [6]. The cytosolic free Ca^{2+} at given total Ca^{2+}, Mg^{2+}, ATP, and EGTA concentrations was calculated using a computer program (WinMAXC 2.50, Stanford University). A detailed description of all methods is available in the Online Data Supplementary.

Data were reported as mean ± SEM. A Student's *t* test, or, when appropriate, one-way ANOVA, was applied to determine statistical significance of the differences. A P value <0.05 was considered statistically significant.

3. Results

3.1. Phosphorylation of sarcoplasmic reticulum Ca^{2+} cycling proteins, PLB and RyRs increases in permeabilized VM when PP and PDE activities are inhibited

Inhibition of protein phosphatase (PP) by Calyculin A (CyA, 0.5 μM) or by CyA plus a broad spectrum PDE inhibitor IBMX (20 μM) markedly increased PLB phosphorylation at a protein kinase A (PKA)-specific Ser^{16} site, detected by Western blots (Fig. 1) and RyR phosphorylation at PKA-dependent Ser^{2809} site, detected by duo-immunolabeling (Fig. 2).

3.2. Periodic, high-power Ca^{2+} signals emerge from stochastic Ca^{2+} sparks when phosphorylation of SR Ca^{2+} cycling proteins becomes increased in response to PP and PDE inhibition or exogenous cAMP

In a free [Ca^{2+}] of 100 nM spontaneous Ca^{2+} sparks in VM are stochastic, non-periodic event of low power in the frequency domain, and of a low amplitude in the space-time domain (Control, Figs. 3A–D). When, in response to PP inhibition by CyA, PKA-dependent PLB phosphorylation is increased (Fig. 1) and the kinetics of SR Ca^{2+} cycling increase, multiple wavelet-like, rhythmic local Ca^{2+} oscillations, i.e. LCRs, emerge (CyA, Fig. 3A and B). When studied in the frequency domain by Fourier analysis, LCRs are synchronized at a dominant frequency of 2.5 Hz (Fig. 3B) and in the space-time domain of the confocal image resulted in high-amplitude individual LCRs Ca^{2+} signals (CyA, Fig. 3C) and summation of these individual Ca^{2+} signals produced a high-amplitude whole-cell (macroscopic) Ca^{2+} signal (ensemble of LCRs) (CyA, Fig. 3D). In other terms, a "Ca^{2+} clock" emerges in VM in a physiologic Ca^{2+} milieu. In the presence of CyA the addition of IBMX, a broad spectrum PDE inhibitor that increases cAMP, and leads to an increase in PKA-dependent phosphorylation [9] (Figs. 1 and 2), further increases the power of the partially synchronized Ca^{2+} signal in the frequency domain (CyA + IBMX, Fig. 3B) and this

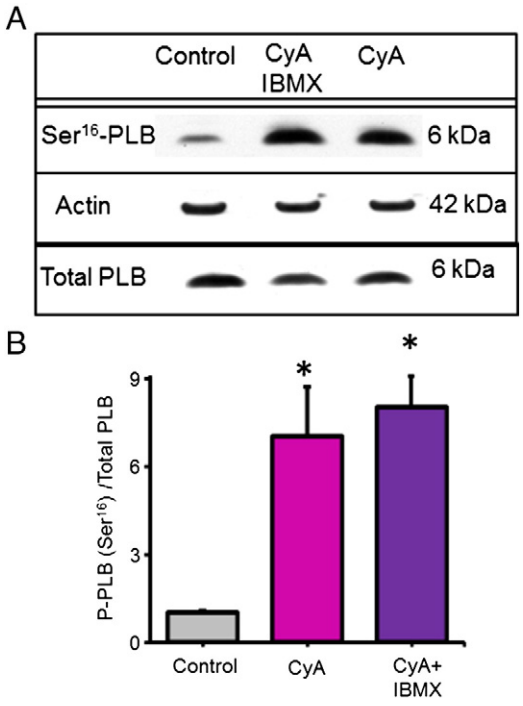

Fig. 1. Enhancement of PLB phosphorylation at a protein kinase A (PKA)-specific Ser^{16} site detected by Western blots in response to PP and PP + PDE inhibition in permeabilized VM. (A) Representative Western blots. (B) Average data of phosphorylated PLB normalized to total PLB in response CyA (0.5 μM) or CyA + IBMX (20 μM) (n = 3 blots). *P < 0.05.