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Original article

Acute reversal of phospholamban inhibition facilitates the rhythmic whole-cell propagating calcium waves in isolated ventricular myocytes

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ABSTRACT

Phospholamban (PLB) inhibits the activity of cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a). Phosphorylation of PLB during sympathetic activation reverses SERCA2a inhibition, increasing SR Ca^{2+} uptake. However, sympathetic activation also modulates multiple other intracellular targets in ventricular myocytes (VMs), making it impossible to determine the specific effects of the reversal of PLB inhibition on the spontaneous SR Ca^{2+} release. Therefore, it remains unclear how PLB regulates rhythmic activity in VMs.

Here, we used the Fab fragment of 2D12, a monoclonal anti-PLB antibody, to test how acute reversal of PLB inhibition affects the spontaneous SR Ca^{2+} release in normal VMs. Ca^{2+} sparks and spontaneous Ca^{2+} waves (SCWs) were recorded in the line-scan mode of confocal microscopy using the Ca^{2+} fluorescent dye Fluo-4 in isolated permeabilized mouse VMs. Fab, which reverses PLB inhibition, significantly increased the frequency, amplitude, and spatial/temporal spread of Ca^{2+} sparks in VMs exposed to 50 nM free [Ca^{2+}]. At physiological diastolic free [Ca^{2+}] (100–200 nM), Fab facilitated the formation of whole-cell propagating SCWs. At higher free [Ca^{2+}], Fab increased the frequency and velocity, but decreased the decay time of the SCWs. cAMP had little additional effect on the frequency or morphology of Ca^{2+} sparks or SCWs after Fab addition. These findings were complemented by computer simulations. In conclusion, acute reversal of PLB inhibition alone significantly increased the spontaneous SR Ca^{2+} release, leading to the facilitation and organization of whole-cell propagating SCWs in normal VMs. PLB thus plays a key role in subcellular Ca^{2+} dynamics and rhythmic activity of VMs.

1. Introduction

The rate at which Ca^{2+} is pumped into the lumen of cardiac sarcoplasmic reticulum (SR) by the SR Ca^{2+}-ATPase (SERCA2a) is tightly controlled by the regulatory protein phospholamban (PLB) [1,2]. Dephosphorylated PLB inhibits SERCA2a activity while increased β-adrenergic stimulation phosphorylates PLB by cAMP–dependent protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), reversing SERCA2a inhibition, thus enhancing the Ca^{2+} uptake into cardiac SR [3,4]. Multiple studies in living ventricular myocytes (VMs) treated with isoproterenol or the monoclonal anti-PLB antibody 2D12 [5,6], or isolated from PLB transgenic and knockout (PLB-KO) mice [7–10] have demonstrated that PLB modulates intracellular Ca^{2+} dynamics, regulating both inotropy and lusitropy.

In diastole, SR Ca^{2+} may be released via cardiac ryanodine receptor channel (RyR2) as Ca^{2+} sparks or spontaneous Ca^{2+} waves (SCWs), a process that is important to physiological rhythm and pathophysiological conditions such as the formation of delayed afterdepolarizations (DADs) and the syndrome of catecholaminergic polymorphic ventricular tachycardia (CPVT) [11–13]. The regulation of spontaneous SR Ca^{2+} release is very complicated, and involves many regulatory factors including both cytoplasmic and lumenal Ca^{2+} [14], multiple protein kinases (e.g., PKA and CaMKII) [15–17], and junctional regulatory protein complexes [18,19]. Within this complex system, the specific role of PLB in the regulation of spontaneous SR Ca^{2+} release during β-adrenergic stimulation in VMs remains unclear. One reason is that besides PLB, β-adrenergic stimulation also phosphorylates RyR2 and other Ca^{2+} handling proteins regulating the SR Ca^{2+} release, making it impossible to

Abbreviations: 2D12, anti-PLB monoclonal antibody; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delay afterdepolarizations; Fab, fab fragment of 2D12; K_{Ca}, Ca concentration for half-maximal effect; PKA, cAMP-dependent protein kinase A; PLB, phospholamban; PLB-KO, PLB deficiency mice; RyR2, cardiac ryanodine receptor channel; SCW, spontaneous calcium wave; SERCA2a, isoform of sarco(endo)plasmic reticulum Ca^{2+}-ATPase in cardiac sarcoplasmic reticulum; SR, sarcoplasmic reticulum; VM, ventricular myocytes.

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delineate whether the reversal of PLB inhibition alone is sufficient to augment spontaneous SR Ca^{2+} release and cause cell-wide SCWs. Previous studies using PLB-KO mice suggest that PLB ablation increases inotropy but not chronotropy [7]. However, the chronic absence of PLB induces multiple adaptive changes of intracellular Ca^{2+} handling proteins [20,21]. The specific role of PLB in rhythmic control in VM remains unclear.

PLB, as a key component of the Ca^{2+} clock, has also been shown to influence rhythmic activity of sinoatrial node cells [22]. Recent studies also indicate that PLB plays a key role in modulating the rhythmic Ca^{2+} activity in VMs. In particular, Kapoor *et al.* demonstrated that expression of Tbx18 induced rhythmic intracellular Ca^{2+} cycling events in VMs, mimicking the "Ca^{2+} clock" of native sinoatrial node cells. In this process, phosphorylation of PLB was 65-fold higher than that in the control VMs, indicating that the modulation of PLB helps to generate rhythmic activity [23]. Sirenko *et al.* also demonstrated that permeabilized VMs showed increased spontaneous Ca^{2+} releases with the self-organized and partial synchronization of Ca^{2+} sparks after PLB inhibition by drugs or 2D12 [24]. On the other hand, Bai *et al.* demonstrated that despite severe SR Ca^{2+} leak with multiple Ca^{2+} sparks or small wavelets, VMs from PLB-KO mice break up the formation of organized and whole-cell propagating SCWs in triggering the DADs [25]. They further showed that PLB ablation actually suppressed triggered activity and stress-induced ventricular tachycardia in the mouse model of PLB-KO plus RyR2 mutation. While these studies point to the regulation of rhythmic Ca^{2+} activity by PLB in VM, an important question that remains to be addressed is whether accelerating SR Ca^{2+} uptake by specifically removing PLB inhibition of the Ca^{2+} pump is pro-arrhythmic (i.e. increasing the automaticity in normal VMs) [24] or anti-arrhythmic (i.e. suppressing the DADs in the CPVT model) [25] in the VMs.

In this study, we took advantage of the specific action of the Fab fragment of the monoclonal anti-PLB antibody 2D12 in blocking the interaction between PLB and SERCA2a in isolated permeabilized (skinned) murine VMs [5,6,26]. We demonstrate that acute and specific reversal of PLB inhibition can significantly increase the frequency, amplitude, and spatial/temporal spread of Ca^{2+} sparks, leading to the facilitation and organization of whole-cell propagating SCWs. These findings were complemented by computer simulations studying the effects of reversal of PLB inhibition in VMs [27,28].

2. Materials and Methods

2.1. Myocyte preparation

The study protocols were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine and the Methodist Research Institute, Indianapolis, Indiana. Hearts from adult C57Bl/6 mice were quickly excised by thoracotomy and retrogradely perfused on a Langendorff apparatus maintained at 37 °C. The enzyme digestion step consisted of perfusing Tyrode's solution containing 1 mg/ml collagenase (Type II, 300 U/mg; Worthington) and 0.1 mg/ml protease (Type XIV, ≥3.5 U/mg; Sigma) for 6 min. Ventricular myocytes (VMs) were dissociated from digested ventricles by gentle mechanical dissociation and used within 3 h. The modified Tyrode's solution contained (in mM) 136 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 1.0 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4 (NaOH) [27]. All chemicals were obtained from Sigma unless indicated otherwise.

2.2. Myocyte permeabilization

VM membranes were permeabilized with saponin (0.005% w/v) for 60 s in a mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 10 HEPES, 0.5 EGTA, and 0.75 $MgCl_2$, pH 7.2 (KOH). Permeabilized VMs were then resuspended in a saponin-free mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 5 KH_2PO_4, 5 MgATP, 10 phosphocreatine, 5 U/ml creatine phosphokinase, 10 HEPES, 0.5 EGTA, 1 $MgCl_2$ (free), 0.015 Fluo-4 (Invitrogen), and 8% w/v dextran (molecular weight ~40,000; to prevent osmotic swelling), pH 7.2 (KOH) [27]. $CaCl_2$ was added to make free [Ca^{2+}] of 50 nM to 1 μM. Free Ca^{2+} concentration and Mg^{2+} concentration were calculated with the use of WebMaxC Extended (maxchelator.stanford.edu). All experiments were performed at room temperature.

2.3. Fab fragment of 2D12 antibody and labeling

Fab fragment of affinity-purified 2D12 was made using a commercial kit (Pierce). In some experiments, 2D12 and Fab were covalently labeled with Alexa-594 (Invitrogen).

2.4. Ca^{2+} spark/wave imaging and immunostaining imaging of the confocal microscopy

We imaged spontaneous Ca^{2+} activity by using the Leica TCS SP8 LSCM inverted microscope fitted with a ×40 1.42 NA oil immersion objective. The Ca^{2+} indicator dye Fluo-4 was excited at 488 nm wavelength with an argon/krypton laser with intensity attenuated to 1–3%. Emission wavelengths >510 nm were detected by the photomultiplier. Fluorescence intensity space–time recordings were acquired in the line-scan mode (1.69 ms/line, 3000 lines/recording) along the longitudinal axis of the myocyte and digitized into 1024 × 1024 pixel images (8-bit) line-scan with nominal pixel dimensions of 98 nm. In some experiments, permeabilized VMs incubated in an internal solution with Fluo-4 were stained with Fab or 2D12 label with Alexa Fluor 594 nm at a concentration of 20 μg/mL for one hour. Samples were then directly examined by microscopy using the ×40 1.42 NA oil immersion objective and a pixel size of 138 nm. The immuno-histological images of Fab-PLB or 2D12-PLB label with Alexa Fluor 594 nm were obtained by illumination with 561 nm laser light, while fluorescence was collected in the long-pass range of >580 nm by the photomultiplier.

2.5. Ca^{2+} spark detection and analysis

The SparkMaster plug-in for ImageJ software [29] was used to detect and analyze Ca^{2+} sparks. The analysis parameters were as follows: scanning speed, 520.8 lines/s; pixel size, 0.08–0.13 μm; spark threshold criteria, 3.8; background, 550–1330; and analysis intervals, 5 [27]. We measured Ca^{2+} spark cluster sizes (spatial widths in line-scan) using a custom algorithm that defines a Ca^{2+} spark cluster as Ca^{2+} sparks separated by less than the single spark average full-width in space and the single spark average full duration in time.

2.6. Ca^{2+}-ATPase assay

Ca^{2+}-dependent ATPase activities of canine SR membranes were measured using an enzyme-coupled spectrophotometric assay [26]. The rate of NADH decay was measured at 340 nm in a SPECTRAmax® PLUS (Molecular Devices) microplate spectrophotometer at 37 °C with 2 μg of membrane protein in buffer containing 50 mM MOPS (pH 7.0), 3 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 3 μg/ml of the Ca^{2+} ionophore A23187, 3 mM ATP, and Ca^{2+}/EGTA as indicated. Ca^{2+}-ATPase activities were measured in the presence and absence of anti-PLB monoclonal antibody 2D12 or the Fab fragment of 2D12. All ATPase activities reported are Ca^{2+}-dependent.

2.7. Computational simulation

We used a VM Ca^{2+} cycling model to simulate the Ca^{2+} sparks and waves in VMs with PLB inhibition [30]. In brief, the model is a three-dimensional Ca^{2+} release unit (CRU) network (65 × 27 × 11 = 19,305 CRUs) with the CRUs coupled via Ca^{2+} diffusion in the myoplasmic space and SR. Each CRU contains a cluster of 100 RyR channels, which were simulated using random Markov transitions. All simulations were