Journal of Molecular and Cellular Cardiology 108 (2017) 8–16

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

β-Adrenergic induced SR Ca^{2+} leak is mediated by an Epac-NOS pathway

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ARTICLE INFO

Article history:
Received 10 February 2017
Received in revised form 26 April 2017
Accepted 27 April 2017
Available online 2 May 2017

Keywords:
Excitation-contraction coupling
Sarcoplasmic reticulum
Ryanodine receptor
Calcium calmodulin-dependent protein kinase
Epac
Nitric oxide synthase

ABSTRACT

Cardiac β-adrenergic receptors (β-AR) and Ca^{2+}-Calmodulin dependent protein kinase (CaMKII) regulate both physiological and pathophysiological Ca^{2+} signaling. Elevated diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) contributes to contractile dysfunction in heart failure and to arrhythmogenesis. β-AR activation is known to increase SR Ca^{2+} leak via CaMKII-dependent phosphorylation of the ryanodine receptor. Two independent and reportedly parallel pathways have been implicated in this β-AR-CaMKII cascade, one involving exchange protein directly activated by cAMP (Epac2) and another involving nitric oxide synthase 1 (NOS1). Here we tested whether Epac and NOS function in a single series pathway to increase β-AR induced and CaMKII-dependent SR Ca^{2+} leak. Leak was measured as both Ca^{2+} spark frequency and tetracaine-induced shifts in SR Ca^{2+}, in mouse and rabbit ventricular myocytes. Direct Epac activation by 8-CPT (8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP) mimicked β-AR-induced SR Ca^{2+} leak, and both were blocked by NOS inhibition. The same was true for myocyte CaMKII activation (assessed via a FRET-based reporter) and ryanodine receptor phosphorylation. Inhibitor and phosphorylation studies also implicated phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) downstream of Epac and above NOS activation in this pathway. We conclude that these two independently characterized parallel pathways function mainly via a single series arrangement (β-AR-cAMP-Epac-PI3K-Akt-NOS1-CaMKII) to mediate increased SR Ca^{2+} leak. Thus, for β-AR activation the cAMP-PKA branch effects inotropy and lusitropy (by effects on Ca^{2+} current and SR Ca^{2+}-ATPase), this cAMP-Epac-NOS pathway increases pathological diastolic SR Ca^{2+} leak. This pathway distinction may allow novel SR Ca^{2+} leak therapeutic targeting in treatment of arrhythmias in heart failure that spare the inotropic and lusitropic effects of the PKA branch.

1. Introduction

β-adrenergic receptor (β-AR) mediated myocyte Ca^{2+} mishandling is commonly described in heart failure (HF) and arrhythmia, which are increasing rapidly [1]. Therefore understanding fundamental mechanisms of β-AR effects on Ca^{2+} handling is critical. β-AR activation is an integral part of the cardiac fight-or-flight response, but its chronic activation (e.g. in HF) contributes to pathological hypertrophic remodeling, contractile dysfunction and arrhythmia.

Acute β-AR activation enhances cardiac contraction (ionotropy) and relaxation (lusitropy), in large part by increasing myocyte Ca^{2+} transients and accelerating $[Ca^{2+}]_i$ reuptake by the sarcoplasmic reticulum (SR). These effects are mainly produced by PKA-dependent phosphorylation of L-type Ca^{2+} channels which increases Ca^{2+} current (I_{Ca}), and phospholamban (PLB) which enhances SR Ca^{2+} uptake and content $[Ca^{2+}]_{SRT}$ (making more Ca^{2+} available for release; Fig. 1, right). The higher Ca^{2+} transient causes stronger contraction, functionally offsetting myofilament Ca^{2+} desensitization by PKA (that otherwise participates in lusitropy) [2]. Studies have also shown that β-AR can also sensitizes ryanodine receptors (RyR) gating and SR Ca^{2+} leak, but while PKA can phosphorylate RyR, the functional effects on RyR are mediated via CaMKII activation and phosphorylation of RyR [3–18]. Chronic CaMKII activation and consequent increase of SR Ca^{2+} is now generally accepted as part of the HF syndrome [19–21]. Indeed, SR Ca^{2+} leak can contribute directly to both diastolic and systolic dysfunction in HF as well as β-AR triggered arrhythmias, via Ca^{2+} wave-induced inward Na^+/Ca^{2+} exchange current (I_{NCX}) that causes delayed afterdepolarizations (DADs), triggered action potentials and premature ventricular contractions (PVCs) [3,4,22,23]. Therefore, inhibition of SR Ca^{2+} leak is a valid therapeutic strategy in HF [19–21] which could improve systolic and diastolic function and limit β-AR-induced arrhythmias.

Two pathways have been independently implicated to mediate β-AR activation of CaMKII and SR Ca^{2+} leak. One pathway involves exchange

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http://dx.doi.org/10.1016/j.yjmcc.2017.04.005
0022-2828/

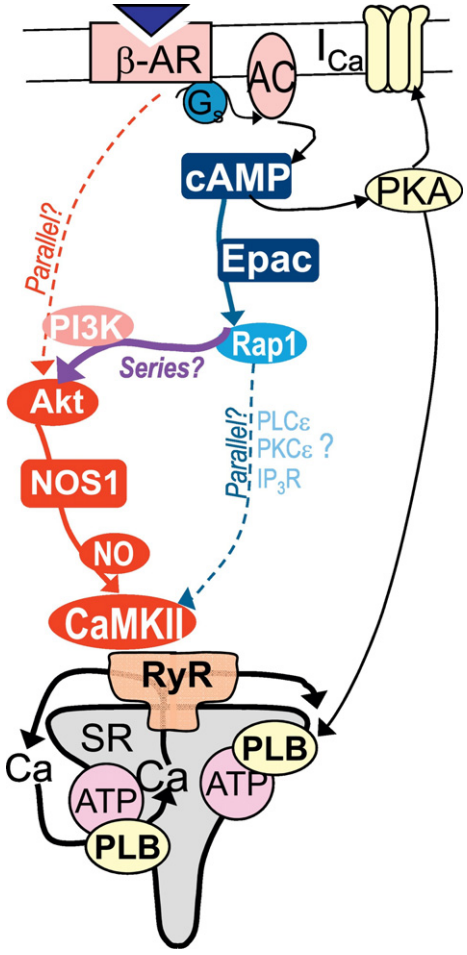

Fig. 1. Proposed pathway for β-AR-induced increase of diastolic SR Ca^{2+} leak. β-AR activation stimulates G-protein (G_s) dependent activation of adenylyl cyclase causing cAMP production that activates both Epac and PKA. The PKA branch enhances Ca^{2+} current (I_{Ca}) and phospholamban (PLB) sensitive SR Ca^{2+}-ATPase (ATP). The Epac branch activates a cascade leading to NOS- and CaMKII-dependent RyR2 phosphorylation that promotes SR Ca^{2+} leak. Broken lines indicate a previously held idea that two parallel pathways might mediate Epac and NOS effects on RyR2.

protein directly activated by cAMP (Epac), a cAMP target parallel to PKA, which may involve some downstream Epac targets leading to CaMKII autophosphorylation and RyR phosphorylation RyR (Fig. 1, blue) [10–18]. Indeed, we have shown that this pathway specifically requires β_1-AR, Epac2 (which localizes at myocyte T-tubules), CaMKIIδ and RyR2 phosphorylation at S2814 [14,16]. For this pathway, it is clear that cAMP is involved and that PKA only contributes indirectly (via PLB-dependent increase in SR Ca^{2+} load), but the details of the pathway from the Epac-Rap1 level to CaMKII are not well resolved (with several mediators implicated, Fig. 1) [10–17].

The other β-AR to CaMKII-RyR pathway involving nitric oxide synthase 1 (NOS1), seemed cAMP-independent, but involving protein kinase B (PKB or Akt) as upstream activators of NOS1dependent CaMKII activation via *S*-nitrosylation (Fig. 1, red) [3–8,24]. But in this case, the steps upstream from the β-AR to Akt were less clearly defined. This pathway was thought to be independent of cAMP and Epac because neither forskolin (direct adenylyl cyclase activator) nor 8-CPT (selective Epac agonist) mimicked β-AR effects [4–6]. This raised the idea of β-arrestin mediated signaling to CaMKII [24], as a parallel pathway from (β-AR to Akt and NOS1; Fig. 1). Recent studies that have revealed the molecular mechanism by which *S*-nitrosylation occurs and mediates CaMKII activation [7,25], localization of NOS1 at the junctional SR domain [9], and robust evidence for cardiac CaMKIIδ in regulating RyR2 have solidified our understanding of the bottom part of the NOS1-RyR pathway.

Here, two labs that helped characterize these two pathways have worked together to further test whether these apparently parallel pathways from β-AR to CaMKII-RyR might be related in series. Much of the work leading to the Epac pathway involved measurement of Ca^{2+} sparks as an index of SR Ca^{2+} leak, often used 8-CPT as an Epac agonist and did not explore NOS involvement. The studies leading to NOS1 involvement in β-AR-induced SR Ca^{2+} leak, more often used the Shannon-Bers method of tetracaine-induced $\Delta[Ca^{2+}]_i$ and $\Delta[Ca^{2+}]_{SRT}$ shifts to measure SR Ca^{2+} leak. Here, we find similar results regardless of SR Ca^{2+} leak method, that 8-CPT (when freshly prepared) mimics the β-AR effects on tetracaine-sensitive SR Ca^{2+} leak, and that NOS mediates Epac-dependent increase of Ca^{2+} sparks, and that Akt is involved. We conclude that these pathways are largely in series (Fig. 1).

2. Materials and methods

2.1. Myocytes isolation

Cardiac myocytes were isolated from New Zealand white rabbits and C57BL6 mice using retrograde Langendorff perfusion using Liberase TM (0.075 mg/ml, Roche) and Trypsin (0.0138%, Gibco) (37 °C) as previously described [26]. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC) in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Ca^{2+} Spark measurements

Spontaneous Ca^{2+} spark were measured in quiescent cardiomyocytes loaded with 5 μM Fluo-4 AM (Molecular Probes) for 30 min [14]. Images were recorded using confocal microscopy in line scan mode (Bio-Rad Radiance 2100, 40× oil immersion objective, 6 ms). Fluo-4 AM was excited at 488 nm using an Argon laser and the emission was collected at 505 nm. All experiments were done in Tyrode's solution (in mM: 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$; pH 7.4 with NaOH). Analysis was made using Firefly, a homemade routine in Python™, which uses analytical criteria similar to other widely used analysis methods [27].

8-CPT is a cAMP analog that is highly selective for Epac vs. PKA activation [28,29]. The 8-CPT concentration used here (10 μM) has no effect on myocyte Ca^{2+} handling or Ca^{2+} sparks in mouse myocytes lacking Epac2 [14] and does not accelerate twitch $[Ca^{2+}]_i$ decline [15], in contrast to very potent effects of PKA to accelerate twitch $[Ca]_i$ decline [14]. Moreover, direct measurements of PKA activity in intact ventricular myocytes showed no significant increase in PKA activation at 10–100 μM 8-CPT [16]. Thus, as used here 10 μM 8-CPT is selective for Epac-vs. PKA-mediated Ca^{2+} handling effects.

2.3. SR Ca^{2+} leak measurements

The protocol used to measure SR Ca^{2+} leak in rabbit was as previously described (Fig. 2) [5]. Briefly, $[Ca^{2+}]_i$ was measured using a fluo-4 (Invitrogen) signal in isolated myocytes in the presence and absence of SR Ca^{2+} leak. Images were recorded using confocal microscopy in line scan mode (Zeiss LSM DUO in Live Channel mode, 40× water immersion objective, 2 ms/line). Tetracaine was used to rapidly and reversibly block the RyR, thus disrupting the SERCA pump-leak balance (Fig. 2). The tetracaine-dependent shift of Ca^{2+} from the cytosol to the SR (decrease in $[Ca^{2+}]_i$ and increase in SR Ca^{2+} content) is proportional to SR Ca^{2+} leak.

Myocytes were subjected to a protocol to load the SR in a graded manner: by emptying the SR with 10 mM caffeine followed either by 30 s of rest, 30 s of rest followed by one single stimulation, or field stimulation at 0.25 to 1.0 Hz. Field stimulations at given rates were