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Original article

Stimulation of NOX2 in isolated hearts reversibly sensitizes RyR2 channels to activation by cytoplasmic calcium

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ABSTRACT

The response of ryanodine receptor (RyR) channels to cytoplasmic free calcium concentration ($[Ca^{2+}]$) is redox sensitive. Here, we report the effects of a mild oxidative stress on cardiac RyR (RyR2) channels in Langendorff perfused rat hearts. Single RyR2 channels from control ventricles displayed the same three responses to Ca^{2+} reported in other mammalian tissues, characterized by low, moderate, or high maximal activation. A single episode of 5 min of global ischemia, followed by 1 min of reperfusion, enhanced 2.3-fold the activity of NOX2 compared to controls and changed the frequency distribution of the different responses of RyR2 channels to calcium, favoring the more active ones: high activity response increased and low activity response decreased with respect to controls. This change was fully prevented by perfusion with apocynin or VAS 2870 before ischemia and totally reversed by the extension of the reperfusion period to 15 min. *In vitro* activation of NOX2 in control SR vesicles mimicked the effect of the ischemia/reperfusion episode on the frequencies of emergence of single RyR2 channel responses to $[Ca^{2+}]$ and increased 2.2-fold the rate of calcium release in Ca^{2+}-loaded SR vesicles. *In vitro* changes were reversed at the single channel level by DTT and in isolated SR vesicles by glutaredoxin. Our results indicate that in whole hearts a mild oxidative stress enhances the response of cardiac RyR2 channels to calcium *via* NOX2 activation, probably by *S*-glutathionylation of RyR2 protein. This change is transitory and fully reversible, suggesting a possible role of redox modification in the physiological response of cardiac RyR2 to cellular calcium influx.

1. Introduction

Ryanodine receptors (RyRs) are high molecular weight tetrameric channels that mediate the release of Ca^{2+} from the sarcoplasmic reticulum (SR) to produce muscle contraction. In cardiac muscle, Ca^{2+} influx through L-type Ca^{2+} channels triggers Ca^{2+} release from the SR through type-2 RyR (RyR2) channels during each action potential. Association of RyR with several proteins, both at the cytoplasmic and at the luminal face of the channel, as well as RyR phosphorylation, modulate RyR channel response to changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]$), the key mechanism of physiological RyR2 activation in cardiac muscle [1].

Considerable evidence gathered over the past 3 decades shows that redox agents modulate RyR activity, presumably through changes in the redox state of a few critical cysteine residues of the protein [2,3]. Oxidants increase, while reducing agents decrease RyR channel activity *in vitro* [4–6]. The role of RyR2 redox modulation *in vivo* is less evident, however. The increased SR Ca^{2+} leak observed in diabetic cardiomyopathy [7,8] and in different models of heart failure [9–11], as well as the arrhythmias observed in a model of sudden cardiac death [12] have been attributed to the oxidation of specific cysteine residues of RyR2, suggesting a pathological role for redox changes in RyR2 channel function. Redox modulation of RyR2, however, may have a physiological role as well. Tachycardia or exercise enhance the generation of reactive species of oxygen (ROS) in heart muscle *via* NOX2, increasing the *S*-glutathionylation of the RyR2 protein and enhancing Ca^{2+} release mediated by RyR2 channels in isolated SR vesicles [13,14]. More recently, Prosser et al. reported that moderate stretch of isolated cardiomyocytes, activates NOX2 at the plasma membrane and generates a burst of Ca^{2+} sparks mediated by RyR2 [15,16]. Detailed aspects, however, of ROS-dependent RyR2 activation remain largely unknown.

Abbreviations: $[Ca^{2+}]$, free Ca^{2+} concentration; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid; ER, endoplasmic reticulum; GSH, glutathione; HEDTA, N-(2-hydroxyethyl)-ethylenediamine-triacetic acid; I5R1, 5 min of ischemia followed by 1 min of reperfusion; I5R15, 5 min of ischemia followed by 15 min of reperfusion; K_a, $[Ca^{2+}]$ for half-maximal channel activation by Ca^{2+}; K_i, $[Ca^{2+}]$ for half maximal channel inhibition by Ca^{2+}; $O_2^-\cdot$, superoxide anion; PC, phosphatidylcholine; P_o, fractional time spent by the channel in the open state; $P_{o\ max}$, maximal theoretical P_o value; POPE, palmitoyl-oleoyl-phosphatidylethanolamine; PS, phosphatidylserine; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; RyR2, type-2 ryanodine receptor; SR, sarcoplasmic reticulum.

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We have previously shown that the endoplasmic reticulum (ER) from rat brain cortex [17–19] and the SR from skeletal muscle [20] contain RyR channels that display, after incorporation in planar lipid bilayers, three different patterns of response to changes in cytoplasmic [Ca^{2+}], namely the low, moderate or high activity responses. Incubation of low activity channels with SH oxidizing agents increases stepwise their response to cytoplasmic [Ca^{2+}], reaching successively the moderate and the high activity responses; conversely, reducing agents have the opposite effect [18,20]. Similarly, the response to cytoplasmic [Ca^{2+}] of RyR2 channels present in the SR from rabbit hearts show marked redox dependence; however, only the moderate or the high activity response were observed [20].

In several tissues, including the heart and the brain, episodes of ischemia and reperfusion enhance the production of ROS [21–23]. Moreover, in a model of whole brain ischemia, RyR channels present in ER from rat brain cortex show enhanced *S*-glutathionylation and increased activation by cytoplasmic [Ca^{2+}] [19]. Up to date, there is no clear evidence of reversible redox modifications leading to modulation of RyR channel activity in the whole heart. Therefore, the aim of this work was to study, in isolated rat hearts, the effects of a single short episode of ischemia/reperfusion on the response of RyR2 channels to cytoplasmic [Ca^{2+}].

2. Material and methods

This study conforms to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH, Publication No. 85-23, revised in 1996), and was approved by the Institutional Ethics Committee of the Faculty of Medicine, University of Chile.

2.1. Langendorff perfused hearts

Rats were anesthetized with pentobarbital (80 mg/kg IP) and heparin 100 U/kg was administered IV. The heart was rapidly excised, mounted in a temperature regulated heart chamber and retrogradely perfused *via* the ascending aorta using a peristaltic infusion pump at a constant flow of 10–14 ml/min with Krebs Henseleit buffer solution (mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 $MgSO_4$, and 11.1 glucose, equilibrated with a gas mixture of 95% O_2/5% CO_2 at 37 °C, pH 7.4. After a stabilization period of 20 min, control hearts were rapidly frozen under liquid nitrogen. Experimental hearts were subjected to 5 min of no-flow global ischemia, followed by either one (I5R1), 5, 10 or 15 min of reperfusion (I5R15), before freezing. In some experiments of I5R1, 0.15 mM apocynin or 10 μM VAS 2870 was added to the perfusion buffer for 10 min before inducing ischemia. Alternatively, hearts were rapidly excised, washed out of blood and SR vesicles were isolated from ventricles without perfusion in the Langendorff system.

2.2. Preparation of cardiac SR-enriched membrane fraction

Frozen ventricles were reduced to powder under liquid nitrogen and homogenized in 4 volumes of 0.3 M sucrose, 30 mM potassium phosphate buffer, pH 7.0, containing protease inhibitors (1 mM PMSF, 1 mM benzamidine, 2 μg/ml leupeptine, 1 μg/ml pepstatin). The homogenate was centrifuged at 5200 ×*g* during 20 min. The pellet was re-extracted as above and the combined supernatants were centrifuged at 16,300 ×*g* for 30 min. The supernatant was recovered and sedimented at 46,800 ×*g* for 45 min. The pellet was resuspended in 0.6 M KCl, 30 mM potassium phosphate buffer pH 7.0 plus protease inhibitors and repelleted at the same speed. The final pellet was resuspended in 0.25 M sucrose, 30 mM imidazole, pH 7.0, plus the above protease inhibitors, and kept frozen in small aliquots at −80 °C.

2.3. Channel recording and analysis

Planar phospholipid bilayers were painted with a mixture of palmitoyl-oleoyl-phosphatidylethanolamine (POPE), phosphatidylserine (PS), and phosphatidylcholine (PC) in the proportion POPE: PS: PC = 5: 3: 2. Lipids obtained from Avanti Polar Lipids (Birmingham, AL) were dissolved in decane to a final concentration of 33 mg/ml. SR vesicles were fused with the bilayer as described previously [17]. During channel recording, the cis compartment (that corresponds to the cytoplasmic compartment) contained 225 mM HEPES–Tris, pH 7.4, 0.5 mM total Ca^{2+} plus sufficient N-(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) and/or ethyleneglycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) to obtain the desired [Ca^{2+}]; required amounts of HEDTA and/or EGTA were calculated with the WinMAXC program (www.stanford.edu/ ~cpatton/wmaxc.zip). The trans compartment (that corresponds to the intrareticular compartment) was replaced with 40 mM Ca–HEPES, 10 mM Tris–HEPES, pH 7.4. Therefore, the charge carrier was Ca^{2+}.

The experiments were carried out at room temperature (22–24 °C), with membranes held at 0 mV. Voltage was applied to the cis compartment, and the trans compartment was held at virtual ground through an operational amplifier in a current-to-voltage configuration. For analysis, data were filtered at 400 Hz (−3 dB) with an eight-pole low-pass Bessel-type filter (902 LPF; Frequency Devices, Haverhill, MA) and digitized at 2 kHz with a 12-bit analog-to-digital converter (Labmaster DMA interface; Scientific Solutions, Solon, OH) with Axotape software (Axon Instruments, Burlingame, CA). Fractional open time (P_o) was computed from records of 30 s or longer with pCLAMP software (Axon Instruments). Channels were classified according to their response to cytoplasmic [Ca^{2+}] as described previously [19,24]. The P_o data as a function of cytoplasmic [Ca^{2+}] were fitted to the following equation:

$$P_o = \left\{\left(P_{o\,max} \times \left[Ca^{2+}\right]^n\right)/\left((K_a)^n + \left[Ca^{2+}\right]^n\right)\right\} \times \left\{K_i/\left(K_i + \left[Ca^{2+}\right]\right)\right\}. \quad (1)$$

In this equation, $P_{o\,max}$ corresponds to the theoretical P_o for maximal channel activation by Ca^{2+}. K_a and K_i correspond to the [Ca^{2+}] for half maximal activation and inhibition of channel activity, respectively, and n is the Hill coefficient for Ca^{2+} activation. The value of $P_{o\,max}$ was fixed to 0.65 for low activity channels and to 1.0 for moderate and high activity channels [18]. Nonlinear fitting was performed with the commercial software SigmaPlot (Systat Software).

2.4. Ca^{2+}-release kinetics

Ca^{2+}-release kinetics was determined in an SX.18MV stopped-flow fluorescence spectrometer (Applied Photophysics Ltd., Leatherhead, U.K.) essentially as described before [25,26], except that Ca^{2+} loading was done at 25 μM $CaCl_2$ in the presence of 2 μg/ml leupeptin and anti-phospholamban antibody (antibody/SR protein = 1/1000). The Ca^{2+} release records were obtained in control conditions, after incubation for 5 min at 30 °C with 250 μM NADPH plus 500 μM glutathione (GSH), or after a second incubation period (5 min at 30 °C) following addition of glutaredoxin (5 IU/ml).

2.5. Determination of NADPH-oxidase activity

Superoxide ($O_2^-\cdot$) production was measured by lucigenin chemiluminescence, as described before [13,14].

2.6. Western blot analysis

RyR2 *S*-gluthationylation: SR vesicles (30 μg protein) were incubated in non-reducing loading buffer plus 20 mM N-ethylmaleimide for