Journal of Molecular and Cellular Cardiology 68 (2014) 38–46

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

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

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ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 25 November 2013
Accepted 31 December 2013
Available online 10 January 2014

Keywords:
Ca^{2+}-induced Ca^{2+} release
Redox signaling
Single channel
Preconditioning
Ischemia/reperfusion

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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0022-2828/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.12.028

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

20 min at 50 °C and separated by SDS-PAGE in 3.5%–8% gradient gels under non-reducing conditions. After electrophoresis and transfer to polyvinylidene difluoride (PVDF) membranes, proteins were probed with the indicated specific antibodies. The bands were quantified by densitometry and the results were normalized with respect to controls run in the same gel. Results are expressed as the ratio anti-GSH/anti-RyR. Anti-GSH antibody was obtained from Virogen (Watertown, MA), anti-RyR antibody was from Thermo Scientific (Rockford, IL), and anti-RyR2 phosphoserine-2814 antibody was from Badrilla Ltd. (Leeds, UK).

Determination of $p47^{phox}$: SR vesicles (10 μg of protein) were subjected to conventional SDS-PAGE, transferred to PVDF membranes and probed with antibody against $p47^{phox}$ from BD Transduction Lab (San Jose, CA).

3. Results

3.1. Response to cytoplasmic [Ca^{2+}] of single RyR2 channels from rat heart ventricles

In this work, we found that RyR2 channels present in SR vesicles isolated from rat heart ventricle exhibited all the responses to cytoplasmic [Ca^{2+}] described for RyR channels from mammalian excitable tissues [20], including the low activity response. Fig. 1 depicts representative current recordings obtained with single RyR2 channels that spontaneously displayed low, moderate or high activity after incorporation in planar lipid bilayers. Low and moderate activity channels increased their P_o in the [Ca^{2+}] range of 3.2 to 32 μM, and decreased P_o at [Ca^{2+}] ≥ 100 μM. Moderate activity channels reached highest P_o values near 0.6, whereas low activity channels displayed highest P_o values about 0.02 (Fig. 1, left and central panels). High activity channels reached P_o values close to 1.0 at 10 μM [Ca^{2+}] and showed no significant inhibition at 500 μM [Ca^{2+}] (Fig. 1, right panel).

The frequencies of emergence of the three different responses to cytoplasmic Ca^{2+} in control ventricles are depicted in Fig. 2A. Of 39 channels recorded in SR vesicles from rat hearts perfused by the method of Langendorff, 13 (33.3%) displayed low activity, 23 (59%) exhibited moderate activity and only 3 (7.7%) displayed the high activity response (Fig. 2A, white bars). This distribution of RyR channel behaviors differs markedly ($p < 0.001$, chi-squared test) from the distribution reported in rabbit cardiac muscle (43% moderate and 57% high activity channels [20]). To test whether the perfusion with the Krebs Henseleit solution was responsible for the emergence of low activity channels and the reduction in the frequency of channels with the high activity response to cytoplasmic [Ca^{2+}], we isolated SR vesicles from the ventricles of rapidly excised rat hearts, following a similar method to that used in rabbit hearts (see Section 2). The distribution of the responses to Ca^{2+} of 21 channels obtained with SR vesicles from rat hearts without perfusion in the Langendorff system is also depicted in Fig. 2A (black bars). The frequencies of appearance of the three channel responses to Ca^{2+} obtained with both SR isolation procedures are not significantly different ($p = 0.741$, chi-squared test).

The detailed responses to cytoplasmic [Ca^{2+}] of RyR2 channels obtained with control rat hearts are shown in Fig. 2B. Low activity channels (filled triangles) displayed $P_o \leq 0.06$ in all the [Ca^{2+}] range tested (0.1–500 μM) and achieved the highest value of $P_o = 0.025 \pm 0.011$ (mean ± SE) in the [Ca^{2+}] range 10–32 μM. Moderate activity channels (open circles) reached $P_o = 0.70 \pm 0.08$ at 32 μM [Ca^{2+}], a value that differs significantly from P_o values displayed by low activity channels at the same [Ca^{2+}] ($p < 0.001$, Student's t-test). High activity channels (filled squares) showed $P_o = 0.95 \pm 0.03$ at 3.2 μM [Ca^{2+}] with no significant inhibition up to 500 μM [Ca^{2+}] ($P_o = 0.90 \pm 0.03$, $p = 0.312$), a behavior that differs markedly from that displayed by moderate and low activity channels. Fitting of P_o values to Eq. (1) (see Section 2.3) yielded the parameters displayed in Table 1. High activity channels had significant lower K_a than moderate and low activity channels, whereas low activity channels displayed lower K_i than moderate activity channels (Table 1).

3.2. Oxidative stress induced in whole ventricles modifies RyR2 channel response

To investigate if the increase in the oxidative status of whole ventricles modifies the frequency of obtaining the different responses to Ca^{2+} in single RyR2 channels, we generated a mild oxidative stress by subjecting isolated hearts to I5R1 in the Langendorff system. A single episode of I5R1 increased 2.3-fold the rate of NADPH-dependent $O_2^-\cdot$ anion generation (Fig. 3A, black bar; $p \leq 0.05$ *vs.* all other conditions). In contrast, 5 min of ischemia alone, without reperfusion (I5R0), did not increase $O_2^-\cdot$ production (Fig. 3A). The generation of $O_2^-\cdot$ returned to basal levels with reperfusion times ≥5 min (Fig. 3A). After 10 min of reperfusion, $O_2^-\cdot$ production was similar to controls (0.96, N = 2; not shown).

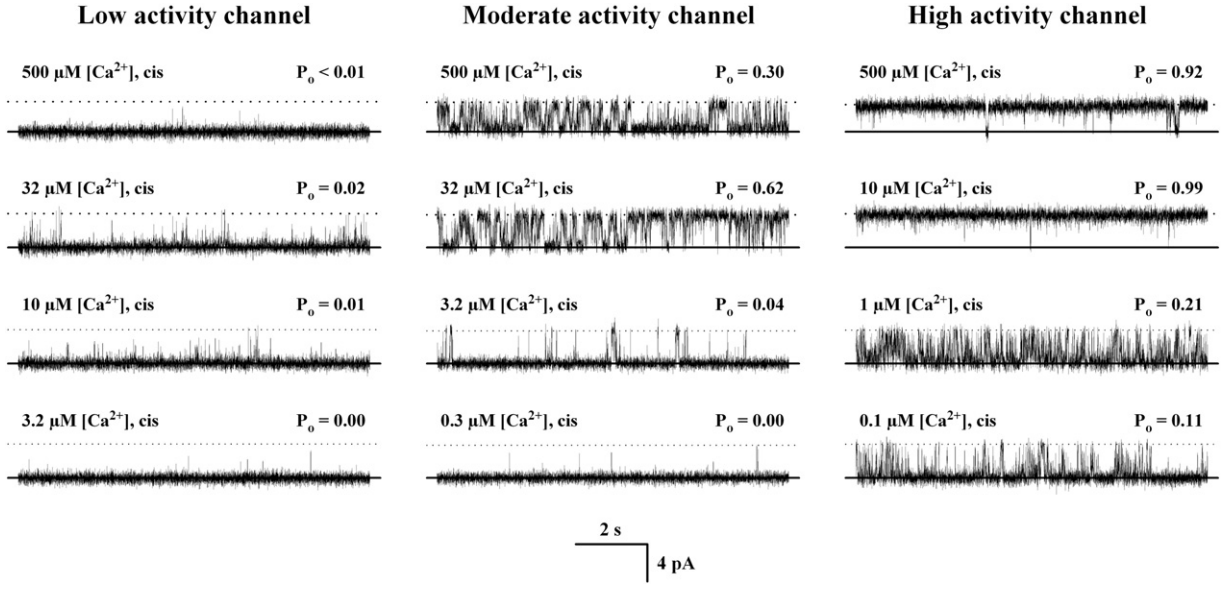

Fig. 1. Single cardiac RyR2 channels display three different responses to cytoplasmic [Ca^{2+}]. Representative current recordings obtained with 3 different single channels that after incorporation in the bilayer spontaneously displayed low, moderate or high activity responses to Ca^{2+}. Free [Ca^{2+}] in the cytoplasmic compartment and average P_o values, calculated from at least 30 s of continuous recordings, are given at top left or right of each trace, respectively. The lipid bilayer was held at 0 mV. Channels open upward.

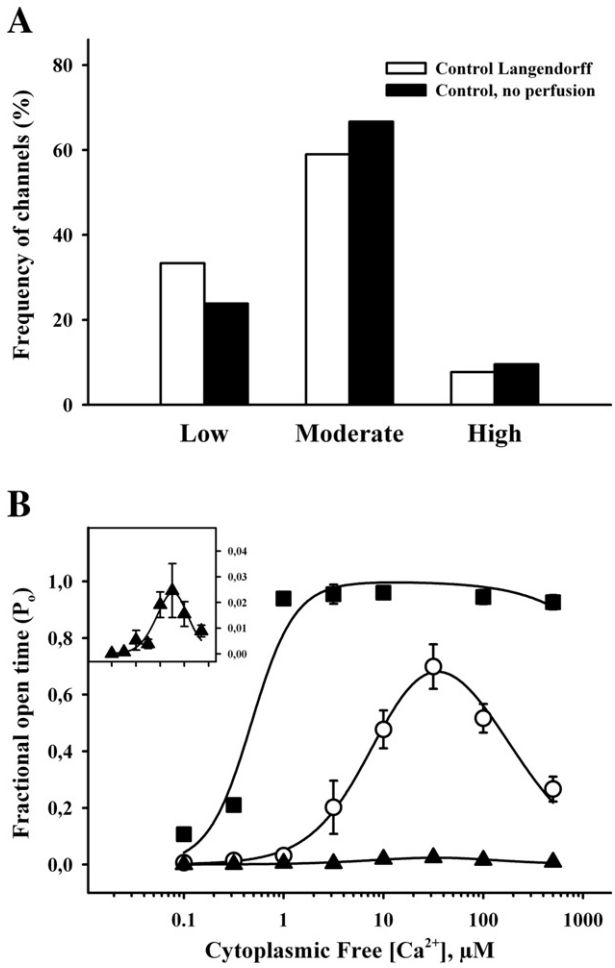

Fig. 2. Activity of single RyR2 channels from control rat hearts. A. Frequency of incorporation of channels with low, moderate, or high activity from control hearts either perfused in the Langendorff system (open bars) or without perfusion (black bars). B. Ca^{2+} response of low (filled triangles), moderate (open circles), and high activity channels (filled squares). Data were obtained with 16 low, 28 moderate and 7 high activity channels. Symbols and error bars depict mean and SE values, respectively. Solid lines represent the best nonlinear fits to Eq. (1) (see Section 2.3). The inset shows the low activity response with amplified vertical scale. Fitted parameters are displayed in Table 1.

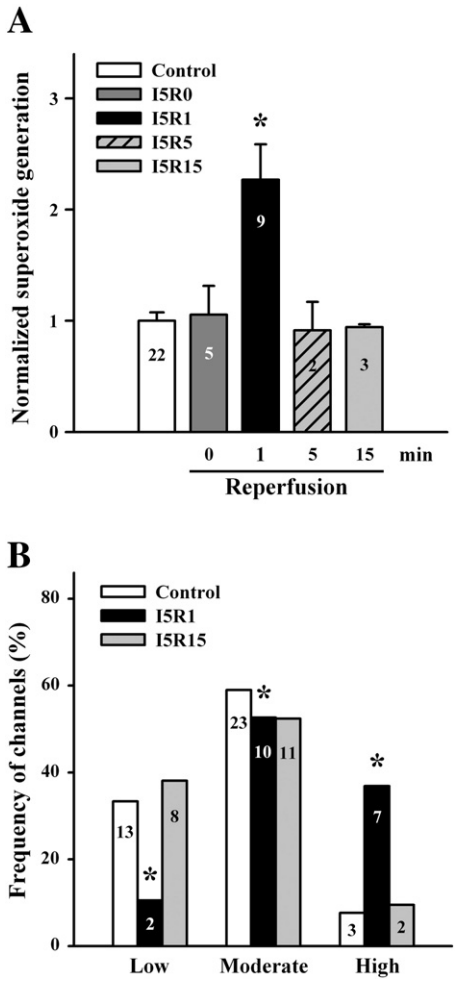

Fig. 3. A single episode of I5R1 promotes $O_2^-\cdot$ anion generation and increases the response of RyR2 channels to cytoplasmic Ca^{2+}. A. Temporal course of $O_2^-\cdot$ generation during reperfusion following a single episode of 5 min of ischemia. Bars depict mean and SE values, respectively; the number on each bar represents the number of experiments in each condition. *: $p < 0.05$ *vs.* all other conditions depicted (one-way ANOVA followed by Tukey-test). B. I5R1 modifies the frequency distribution of channel responses to Ca^{2+} and 15 min of reperfusion reverses the effect to the control distribution. *: $p < 0.05$ *vs.* all other conditions depicted (chi-squared test).

In addition, I5R1 produced a significant shift in the frequency distribution of RyR2 channel responses to cytoplasmic $[Ca^{2+}]$ to higher activity responses: the frequency of channels with high activity increased, whereas low activity channels decreased (Fig. 3B, black bars, $p = 0.011$ *vs.* control). I5R1, however, did not add a new response of RyR2 to Ca^{2+}, similar to the effect of 5 min of ischemia in rat brains [19], since data obtained with moderate and high activity channels from I5R1 hearts are well described by the functions fitted to control channel data (see Fig. S1, black symbols and dotted lines, respectively). Moreover, K_a of high activity channels from ischemic hearts (0.34 ± 0.05) were similar to that of control hearts (0.48 ± 0.07, see Table 1; $p = 0.132$, chi-squared test). The parameters of moderate activity channels from I5R1 hearts could not be obtained, since P_o for only 2 different $[Ca^{2+}]$ were obtained in this case (see Fig. S1, black circles).

Table 1
Fitting parameters of the three responses to cytoplasmic $[Ca^{2+}]$ of single RyR2 channels.

	K_a (µM)	n_{Hill}	K_i (µM)
Low	60 ± 11[a]	1.3[b]	4.4 ± 0.9[a]
Moderate	9.4 ± 0.8[a]	1.3 ± 0.3	141 ± 17[a]
High	0.48 ± 0.07[a]	2[b]	5000[b]

Parameter values ± SE were obtained by fitting to Eq. (1) P_o mean values obtained with single channels from control hearts that displayed low, moderate or high activity responses (see Section 2.3 and legend to Fig. 2B).
[a] $p < 0.005$ *vs.* other response types (Student's t-test).
[b] Parameter was fixed to the indicated value for data fitting (see Section 2.3).

The changes in the frequencies of single RyR2 channel responses were transitory and readily reversible, since after 15 min of reperfusion the distribution of channel responses did not differ from control values ($p = 0.883$), but differed significantly from I5R1 ($p = 0.042$, Fig. 3B, gray bars). Again, only the frequency of RyR2 responses changed, since data of low and moderate channels from I5R15 are adequately described by the functions obtained with channels from control data (see Fig. S1, gray symbols and dotted lines, respectively). K_a and K_i (10.4 ± 0.1 and 162 ± 2 µM $[Ca^{2+}]$, respectively) of moderate activity channels from I5R15 hearts did not differ from the corresponding values of control hearts (see Table 1; $p > 0.25$). Parameter of low activity channels from hearts subjected to I5R15 could not be calculated, since scarce data were collected in the range where P_o changed with cytoplasmic $[Ca^{2+}]$ (see Fig. S1, gray triangles).

The generation of $O_2^-\cdot$ anion was significantly reduced when the hearts were perfused for 10 min prior to I5R1 with 0.15 mM apocynin, a NOX2 inhibitor, or with 10 µM VAS 2870, a general NOX inhibitor (Fig. 4A). Both inhibitors, prevented the translocation of $p47^{phox}$ to the membrane of cardiomyocytes in whole ventricles (Fig. 4B), thus

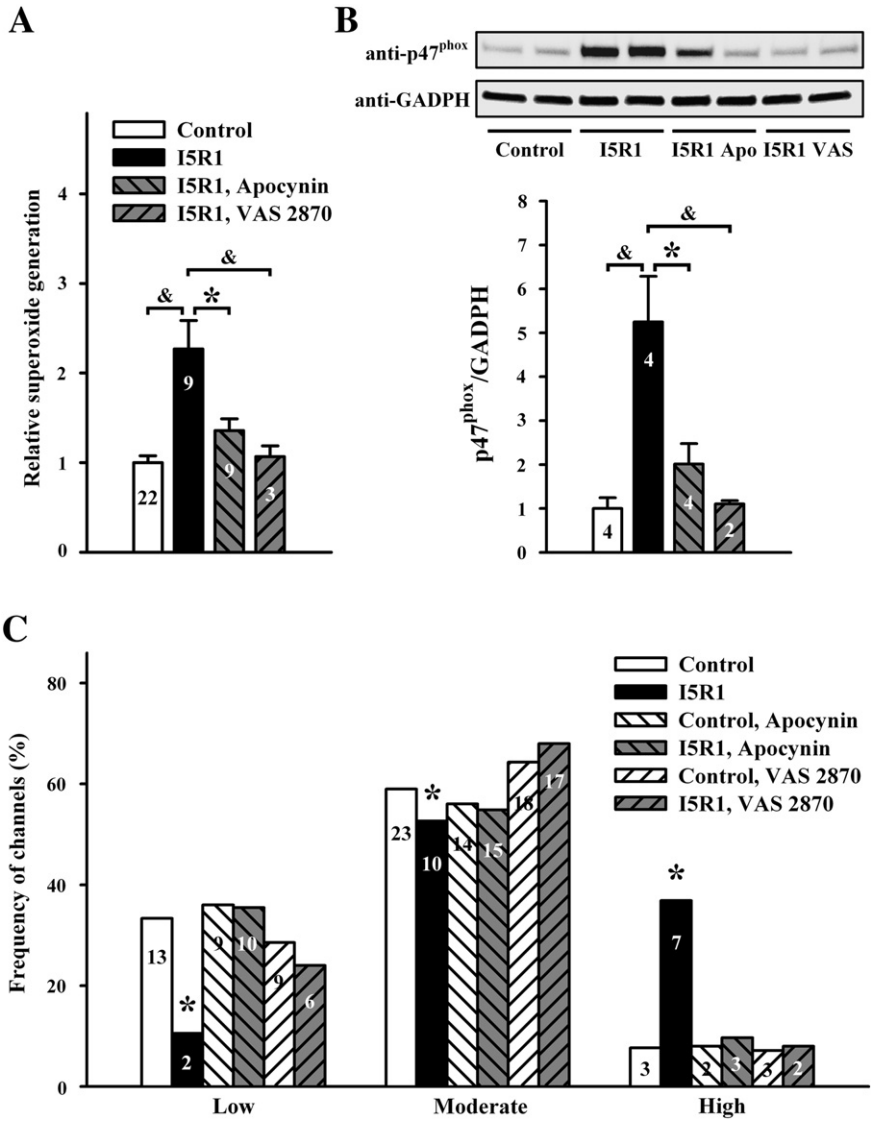

Fig. 4. Apocynin and VAS 2870 prevent NOX2 activation and the enhanced response of RyR2 channels to cytoplasmic Ca^{2+} induced by a single episode of I5R1. A. I5R1 promotes $O_2^-\cdot$ generation that is prevented by 10 min of perfusion with 0.15 mM apocynin or 10 μM VAS 2870 prior to I5R1. &: $p < 0.01$; *: $p \leq 0.05$ (one-way ANOVA followed by Tukey-test). B. Apocynin or VAS 2870 prevents translocation of $p47^{phox}$ to the membrane. &: $p < 0.01$; *: $p \leq 0.05$. C. Apocynin or VAS 2870 prevents the change in the frequency distribution of RyR2 responses to Ca^{2+}. Effect of I5R1 is compared against their respective controls. *: $p < 0.05$ (chi-squared test). No difference among controls was found.

inhibiting NOX2 activation *ex vivo*. Fig. 4C shows that perfusion with apocynin or VAS 2870 before I5R1, fully prevented the changes in RyR2 channel behavior, since no difference between I5R1 plus NOX inhibitor, compared with their respective control, was found (Fig. 4C, hatched gray bars, $p = 0.976$ or 0.866 with apocynin or VAS, respectively). Therefore, I5R1 favored RyR2 responses with lower K_a for activation by $Ca^{2\pm}$ and higher K_i for inhibition by Ca^{2+}, probably as a result of a change in the redox state of the RyR2 channel protein [20], at least *via* NOX2.

3.3. Redox modification of RyR2 in whole ventricles and in vitro

We have shown before that stimulation of NOX2 in dog hearts generates $O_2^-\cdot$ anion and promotes *S*-glutathionylation of RyR2 [14]. Here, we show that I5R1 increased *S*-glutathionylation of RyR2 by 1.7-fold, a change that was prevented by prior perfusion with apocynin (Figs. 5A and B). Since CaMKII is also subject to redox modulation and enhances activation of RyR2 by Ca^{2+} [27], we tested if I5R1 modified the phosphorylation level of serine 2814 by Western blot analysis. I5R1 did not change the phosphorylation level of RyR2 (Figs. 5C and D).

As a next step, we tried to reproduce *in vitro* the redox changes elicited by I5R1. Therefore, we incubated SR vesicles from control rat hearts with NADPH plus GSH or with GSNO, two procedures that induce *S*-glutathionylation of RyR2 [14] or RyR1 [28], respectively. Remember, however, that GSNO is also able to *S*-nitrosylate the RyR channel protein. Incubation with NADPH plus GSH before incorporation of RyR2 channels to the bilayer significantly modified the frequency of emergence of the different channel responses to Ca^{2+}, as shown in Fig. 6 (black bars, $p = 0.016$ *vs.* control), which mimicked the distribution of channel responses observed after I5R1 (compare with Fig. 3B, black bars). Similar pattern of RyR2 responses was obtained after incubation with GSNO (Fig. 6, gray bars, $p = 0.017$ *vs.* control).

3.4. Reversion of RyR2 changes

Fig. 7A displays typical current recordings of a single RyR2 channel, obtained from SR vesicles incubated with NADPH plus GSH prior to channel incorporation to the bilayer. The channel displayed moderate activity, as evidenced by a P_o value of 0.42 at 10 μM [Ca^{2+}] (Fig. 7A, upper trace). Seventy seconds after addition of 1 mM dithiothreitol (DTT) to the cytoplasmic compartment, P_o decreased to 0.03, a value

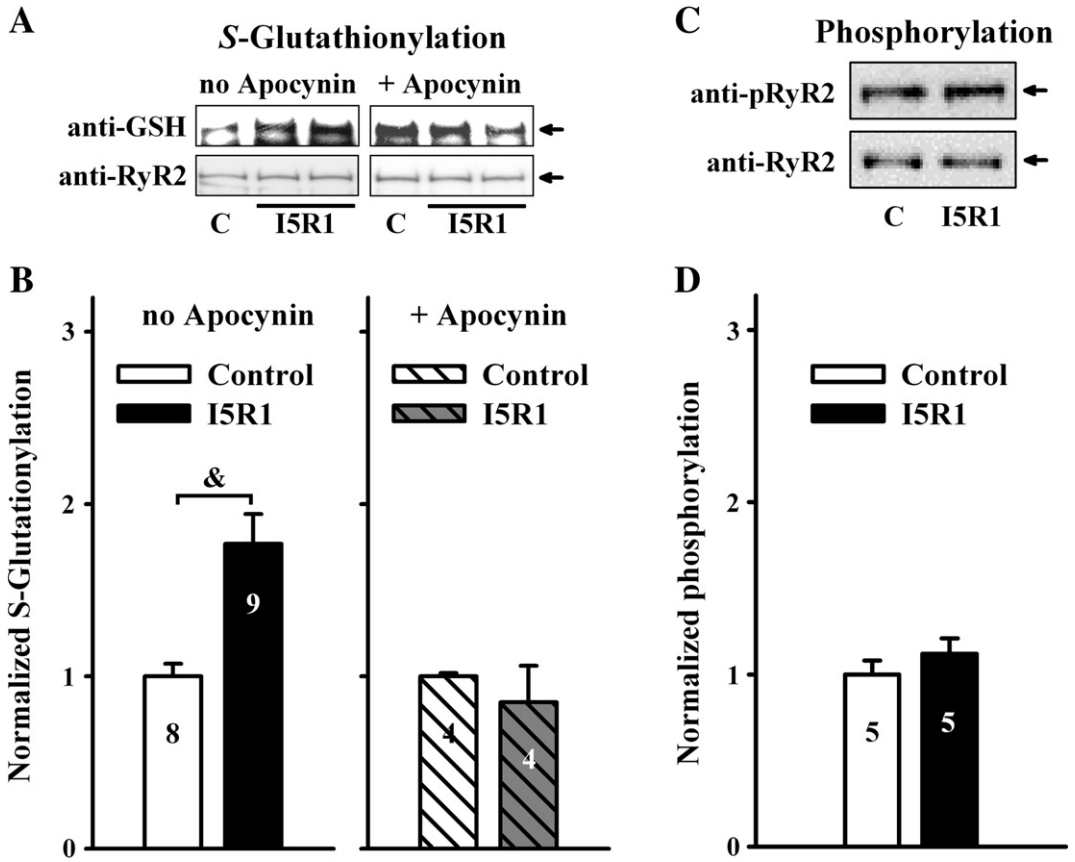

Fig. 5. I5R1 enhances *S*-glutathionylation, but not phosphorylation of RyR2. A: Representative Western blots in SR vesicles obtained from control (C) or I5R1 ventricles without (left blots) or with (right blots) perfusion with apocynin were probed with anti-GSH antibody (upper blots) or anti-RyR2 antibody (lower blots) as detailed in Section 2.6. B: Mean and SE values of normalized *S*-glutathionylation (anti-GSH/anti-RyR2 isoform) calculated from blots like those shown in A.; &: $p < 0.01$ (Student's t-test). C. Representative Western blots in SR vesicles obtained from control or I5R1 ventricles were probed with anti-Pser2814 RyR2 antibody (anti-pRyR2, upper blot) or anti-RyR2 antibody (lower blots). Arrows indicate the position of RyR2 in the blots. D. Normalized phosphorylation of RyR2 obtained from experiments similar as in C. In A and C, arrows indicate the position of RyR2 in the respective blots. In B and D, the number on each bar represents the number of experiments for each condition.

seen only in low activity channels at this [Ca^{2+}] (see Fig. 2B), indicating that DTT changed the response of the channel to Ca^{2+} from the moderate to the low activity response. Fig. 7B depicts representative recordings of a single channel present in SR vesicles isolated from a heart subjected to a single episode of I5R1. The channel displayed the high activity response after incorporation in the bilayer, with P_o of 0.95 and 0.97 at 500 and 10 μM cytoplasmic [Ca^{2+}], respectively (Fig. 7B, upper traces). After 2 min of incubation with 0.5 mM DTT followed by extensive perfusion of the cytoplasmic compartment with DTT-free buffer, the channel displayed the moderate response to cytoplasmic Ca^{2+} (Fig. 7B, lower traces). Therefore, high activity channels that appeared with higher frequency after I5R1 changed their response to cytoplasmic [Ca^{2+}] by incubation with DTT in the bilayer setup. The experiments exemplified in Fig. 7 show that the enhanced response to Ca^{2+} of RyR2 channels from ventricles subjected to one episode of I5R1, or produced by incubation of control SR vesicles in conditions that oxidize RyR2 cysteine residues, were reversed by DTT in the bilayer setup. These results favor the idea that functional changes in RyR2 activity result from reversible redox modification(s) of RyR2.

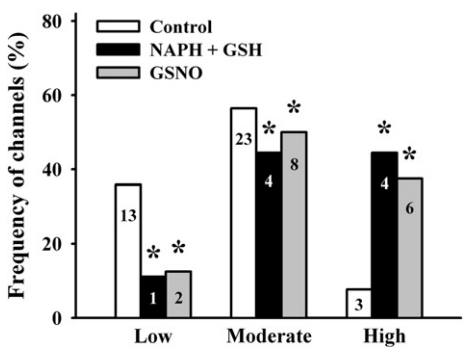

Fig. 6. Incubation of cardiac SR vesicles with NADPH plus GSH or with GSNO mimics the frequency distribution of RyR2 channel responses to Ca^{2+} induced by I5R1. *: $p < 0.05$ *vs.* control condition (chi-squared test).

In addition, we investigated the enzymatic reversibility of the changes in RyR2 channel function induced by the redox changes promoted by incubation of cardiac SR with NADPH plus GSH. To this aim, we determined the effect of glutaredoxin, a thiol oxidoreductase, on Ca^{2+} fluxes in Ca^{2+}-loaded SR vesicles. Since Ca^{2+} fluxes are the result of the average P_o of a population of channels (although heterogeneous in regard of their response to cytoplasmic Ca^{2+}), this approach is an easier and more efficient complementary method than single channel studies to assess the reversibility in RyR2 channel function. Fig. 8A shows Ca^{2+} release kinetics in control SR vesicles before and after 5 min incubation with NADPH plus GSH; the ROS produced *in vitro* by NOX2 greatly enhanced Ca^{2+} release kinetics. Incubation of these vesicles with glutaredoxin fully reversed Ca^{2+} release to the control condition (Fig. 8B). Incubation with NADPH plus GSH did not enhance Ca^{2+} release in SR vesicles incubated with 50 μM ryanodine for 30 min before the release measurement, indicating that the increased Ca^{2+}-release rate induced by NOX2-derived ROS production occurred *via* RyR2 (Fig. 8C). On average, the rate of Ca^{2+} release increased 2.2-fold after

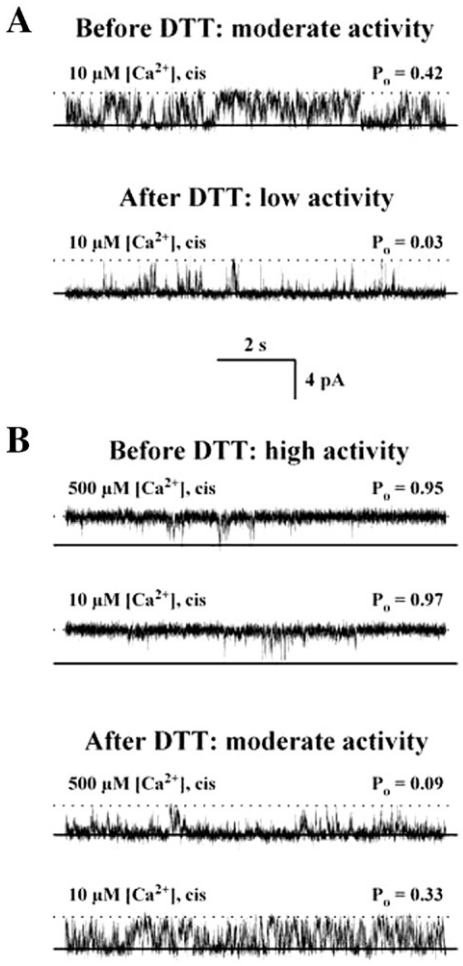

Fig. 7. DTT decreases the response to cytoplasmic Ca^{2+} of cardiac RyR2 channels. Current traces were obtained with two different single channels before and after incubation with DTT in the bilayer setup. Free [Ca^{2+}] in the cytoplasmic compartment and average P_o values from the entire recordings (>30 s) are depicted at the top left or right of each trace, respectively. A. Channel obtained from control SR vesicles incubated with NADPH plus GSH before incorporation in the bilayer: DTT (1 mM) changed the channel response from moderate (upper trace) to low activity (lower trace). B. A channel from ventricles subjected to I5R1: the channel displayed initially the high activity response as exemplified in the upper traces; after incubation with DTT the channel switched to the moderate response (lower traces).

incubation with NADPH plus GSH ($p < 0.05$), whereas further incubation with glutaredoxin decreased Ca^{2+} release to rates that did not differ from control (Fig. 8D). Similar results were obtained with thioredoxin (data not shown).

4. Discussion

The main result of the present work is that exposure of whole ventricles *ex vivo* to a single 5 min episode of ischemia followed by 1 min of reperfusion increased NOX2 activity, which in turn led to redox-dependent enhancement of cardiac RyR2 response to cytoplasmic [Ca^{2+}]. We have previously shown that brief episodes of tachycardia or exercise, which produce myocardial preconditioning in living dogs, stimulate NOX2 and enhance RyR2 channel activity from dog hearts [13,14]. Moreover, *in vivo* administration of the NOX2 inhibitor apocynin abolishes the preconditioning effects of exercise and tachycardia [13,14]. In a similar way, *ex vivo* perfusion of isolated rat hearts with apocynin or VAS 2870 prior to I5R1 prevented: a) NOX2 activation, as indicated by the inhibition of $p47^{phox}$ translocation to the membrane, and b) the redox-dependent increase of RyR2 channel activity in the present work. Conversely, activation of NOX2 *in vitro*, by incubation of SR vesicles from control hearts with NADPH plus GSH: a) effectively mimicked the pattern of responses to Ca^{2+} from RyR2 channels after I5R1, as verified at the single channel level, and b) increased Ca^{2+} release rates measured in isolated SR vesicles.

The enhanced response of single RyR2 channels to cytoplasmic [Ca^{2+}], induced *ex vivo* by I5R1 or *in vitro* by activation of NOX2, was reversed by DTT in the bilayer setup. Moreover, incubation with glutaredoxin or thioredoxin reversed the enhanced Ca^{2+} release rates from SR vesicles caused by activation of NOX2 *in vitro*. Extension of the reperfusion period to 15 min completely reversed the increased response of cardiac RyR2 channels produced during I5R1, indicating that in whole ventricles the redox changes induced in RyR2 *via* NOX2 stimulation during I5R1 are transitory and reversible, an important requirement for considering redox modification of RyR2 channels as a physiological regulatory response. Based on these results, we propose that cardiomyocytes express *in vivo* both oxidative and reducing pathways that participate in the regulation of the redox state of critical

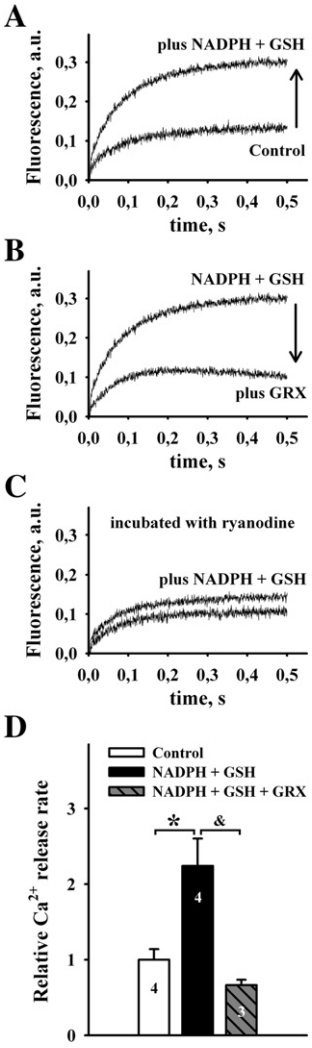

Fig. 8. Increase in Ca^{2+} release by ROS generation *in vitro* and reversion by glutaredoxin. SR vesicles (1 mg/ml) actively loaded with Ca^{2+} were mixed (1:10) in a stopped flow fluorescence spectrometer with a solution that produced upon mixing 1 μM [Ca^{2+}] and 1.2 mM free [ATP]. Ca^{2+} release was measured with Calcium Green 5N. A. Representative fluorescent records illustrating Ca^{2+} release in control conditions, or after incubation with NADPH plus GSH. B. Ca^{2+} release in vesicles incubated with NADPH plus GSH, or after incubation of the same vesicles with glutaredoxin. C. Ca^{2+} release in SR vesicles incubated with 50 μM ryanodine for 30 min before and after incubation with NADPH plus GSH. D. Bars show the relative rate of Ca^{2+} release in control conditions, after incubation with NADPH plus GSH, and after further incubation with glutaredoxin. *: $p < 0.05$; &: $p < 0.01$ (one-way ANOVA followed by Tukey-test).

cysteine residues of RyR2, which in turn tune their response to cytoplasmic [Ca^{2+}] at different physiologic and/or pathologic conditions.

ROS generation is critical to induce cardioprotection by ischemic [29,30] or pharmacologic stimuli [31]. Although a limited amount of ROS is produced during preconditioning ischemia, ROS generated during reperfusion is responsible for cardioprotection [32]. Both mitochondria and NOX2 have been proposed as the source of ROS in ischemic preconditioning; indeed, preconditioning is impaired in NOX2 KO mice [33]. Here we show NOX2 activation after I5R1, but not after 5 min of ischemia with no reperfusion (see Section 3.2); therefore, the first minute of reperfusion seems to be crucial for NOX2 activation. Since NOX2 is activated reversibly in response to stretch in isolated cardiac myocytes [15,16] and perfusion of isolated hearts with an hypoosmotic solution favored the translocation of $p47^{phox}$ to the membrane, activating NOX2 and increasing $O_2^-\cdot$ production (see Fig. S2), we propose that NOX2 activation in our present experiments occurs following the plasma membrane stretch produced by osmotic cell swelling in the early period of reperfusion, caused by accumulation of intracellular ions and metabolites during ischemia.

The present results indicate that NOX2 is an essential part of the pathway that leads to the increased response of RyR2 to cytoplasmic [Ca^{2+}] during I5R1 and favor the idea that the change in RyR2 activity is due to *S*-glutathionylation of the channel protein, but not through CaMKII-dependent phosphorylation. *S*-glutathionylation of RyR2 and enhancement of RyR channel activity have been reported after 5 min of ischemia in rat brain cortex [19] and after five episodes of 5 min of tachycardia or exercise with intervening 5 min rest periods in the dog heart [13,14]. Nevertheless, association of one particular cysteine redox modification with any physiological effect does not necessarily imply causality between them. For example, although ischemia induces both *S*-nitrosylation and *S*-glutathionylation of RyR2 in rat brain cortex, only *S*-glutathionylation but not *S*-nitrosylation of RyR2 enhances channel response to Ca^{2+} *in vitro* [19].

Here, we report for the first time that cardiac RyR2 channels also exhibit the low activity response to cytoplasmic Ca^{2+}. This result is not surprising, however, since low activation by Ca^{2+} is the most frequent response of RyR channels from rat brain cortex, a tissue that expresses RyR2 as the major RyR isoform [19,20]. At present, the response of cardiac RyR2 channels is usually described as a sigmoidal activation at low [Ca^{2+}], with scant inhibition at mM [Ca^{2+}] (high activity channels) [34–37]. Yet, channels displaying the moderate response to Ca^{2+} have been described in mammalian hearts [20,38], and inhibition at high [Ca^{2+}] has been recognized as an extremely labile property in cardiac RyR2 [39], a fact that probably reflects the presence of highly reactive cysteine residues in RyR2 protein. Our present results indicate that the emergence of low activity channels in rat cardiac muscle did not result from the Langendorff perfusion method; hence, we cannot discard species differences in the response to Ca^{2+} of cardiac RyR2 channels from rabbit or rat.

The observation that RyR2 channels from control rat hearts displayed the low activity response to Ca^{2+} with less frequency than RyR channels from control rat brain cortex [19], may reflect a less reduced intracellular environment in cardiac tissue in comparison to brain tissue [40]. If RyR2 channels from rat cardiac muscle have similar modulation by cytoplasmic [Mg^{2+}] and [ATP] as reported for RyR channels from rat brain cortex, only channels with the high activity response would promote Ca^{2+}-induced Ca^{2+} release in the rat heart under physiological [Mg^{2+}] and [ATP] [18,19]. Due to their weak response to cytoplasmic Ca^{2+}, RyR2 channels with low activity (with less redox-modified cysteine residues) would not participate in Ca^{2+}-induced Ca^{2+} release and would rather represent quiescent channels. In other physiological conditions, however, such as increased preload [15,16], and after tachycardia or exercise [13,14] quiescent RyR2 channels would be transformed into moderate or high activity channels by redox modification, thus favoring the increase of stroke volume and cardiac output. Therefore, changes in RyR2 redox state would recruit more or less quiescent channels, depending on the prevailing physiological conditions that could vary from beat to beat [15].

Disclosures

None declared.

Funding

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico, Grants 1110257 and 1130407. The funding agency had no participation in study design; in collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the article for publication.

Acknowledgments

The authors thank Dr. Cecilia Hidalgo for her critical reading of the manuscript. The technical assistance of Guillermo Arce and Rodrigo Durán is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.12.028.

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