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Review

Redox regulation of cardiac calcium channels and transporters

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Abstract

Intracellular concentrations of redox-active molecules can significantly increase in the heart as a result of activation of specific signal transduction pathways or the development of certain pathophysiological conditions. Changes in the intracellular redox environment can affect many cellular processes, including the gating properties of ion channels and the activity of ion transporters. Because cardiac contraction is highly dependent on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and $[Ca^{2+}]_i$ regulation, redox modification of Ca^{2+} channels and transporters has a profound effect on cardiac function. The sarcoplasmic reticulum (SR) Ca^{2+} release channel, or ryanodine receptor (RyR), is one of the well-characterized redox-sensitive ion channels in the heart. The redox modulation of RyR activity is mediated by the redox modification of sulfhydryl groups of cysteine residues. Other key components of cardiac excitation–contraction (e–c) coupling such as the SR Ca^{2+} ATPase and L-type Ca^{2+} channel are subject to redox modulation. Redox-mediated alteration of the activity of ion channels and pumps is directly involved in cardiac pathologies such as ischemia–reperfusion injury. Significant bursts of reactive oxygen species (ROS) generation occur during reperfusion of the ischemic heart, and changes in the activity of the major components of $[Ca^{2+}]_i$ regulation, such as RyR, Na^+–Ca^{2+} exchange and Ca^{2+} ATPases, are likely to play an important role in ischemia-related Ca^{2+} overload. This article summarizes recent findings on redox regulation of cardiac Ca^{2+} transport systems and discusses contributions of this redox regulation to normal and pathological cardiac function.
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1. Introduction

Changes in the reduction/oxidation (redox) state of proteins play an important role in many cellular functions including immunological host defense, gene transcription, cellular metabolism, ionic homeostasis and signal transduction. Typically, sulfhydryl (SH) groups of cysteine residues are potential targets for redox modification of proteins. Alteration in the redox state of SH groups of two neighboring cysteine residues can lead to formation or braking of disulfide bonds. This redox modification of disulfide bonds affects the structure and function of ion regulatory proteins including ion channels, pumps and transporters. It is well established that the activity of various ion regulatory proteins can be modulated by redox-dependent mechanisms [1–3]. Dependent on the type of channel, pump and other transporters, the redox modification has a variety of different functional consequences. For example, reagents that oxidize thiols activate cardiac ryanodine receptor (RyR) channels [4] (see Table 1), but inhibit the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) [5]. The specificity of redox modulation depends on many factors including the redox state of the intracellular (micro-)environment, and concentration and nature of redox active molecules. At low levels these redox active molecules constitute a basal endogenous redox buffering system that reversibly interacts with specific cellular targets and generates an environment tailored towards optimal performance of ion channels and transporters. At high concentrations, however–often encountered transiently or permanently under pathological conditions–redox active species are capable of irreversibly modifying enzyme function or even damaging proteins.

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Table 1
Effects of the redox modification of sulfhydryl groups of cardiac Ca^{2+} channels and transporters

	Effects of compounds that oxidize SH groups	Effects of compounds that reduce SH groups	Effects of compounds that *S*-nitrosylate SH groups
Ryanodine receptor (RyR) Ca^{2+} release channel	Thiol oxidizing agents (thimerosal, DTDP, DTNB) in submillimolar concentrations increased RyR channel activity [47,52,53] ROS ($O_2^{-\cdot}$ and H_2O_2) increased the open probability of RyR [4,58,61,63] ROS caused initial stimulation, followed by inactivation of RyR [64]	Reagents that reduce thiols (β-mercaptoethanol, DTT, GSH and NADH) decreased RyR activity [53,57,61]	*S*-nitrosothiols (CysNO 0.1 mM; GSNO 1 mM and SNAP 1 mM) and NO/$ONOO^-$ donor SIN-1 (0.2 mM) stimulated channel activity. The effect was reversed by DTT [44,70] L-arginine-derived NO inhibited RyR channel activity [71]
Voltage-dependent L-type Ca^{2+} channel	Thiol oxidizing agents (thimerosal, PCMBS, PHMPS, MTSEA) decreased both I_{Ca} and I_{Ba} through α_{1C} subunit; effect was reversed by DTT [92–94] ROS generated from X+XO or H_2O_2 decreased I_{Ca} [95,96] Thiol oxidizing agent (DTNB; submillimolar concentration) stimulated I_{Ca} [97]	DTT (1–2 mM) did not affect I_{Ca} [92,94]. Millimolar concentrations of DTT and GSH inhibited I_{Ca} [97]	CysNO, GSNO and SNAP inhibited I_{Ba} [93] CysNO, GSNO and SIN-1 (all 1 mM) stimulated I_{Ca} [97]
SR Ca^{2+} ATPase (SERCA)	Reagents that oxidize thiols inhibited SERCA activity [99,100] ROS ($O_2^{-\cdot}$ and H_2O_2) inhibited SR Ca^{2+} uptake [5,58,100–103]	DTT and GSH protected against thiol oxidant effects [99]	*S*-nitrosothiols did not affect SERCA activity [70,74,104] $ONOO^-$ (10–50 μM) increased activity of SERCA2 [104]
Na^+–Ca^{2+} exchange (NCX)	ROS generated from X+XO and H_2O_2 enhanced NCX-mediated Ca^{2+} fluxes [109,110,112,113] GSH/GSSG (1–2 mM) stimulated NCX [109] Strong oxidant HOCl inhibited NCX [110]	DTT and GSH did not affect NCX [111]	
Plasmalemmal Ca^{2+} ATPase (PMCA)	ROS generated from X+XO and H_2O_2 decreased PMCA activity [105,106]		

CysNO: *S*-nitrocysteine; DTDP: 2.2′-dithiodipyridine; DTNB: 5.5′-dithio-bis(2-nitrobenzoic acid); DTT: dithiothreitol; GSH: glutathione; GSNO: *S*-nitrosoglutathione; I_{Ba}: barium current; I_{Ca}: calcium current; MTSEA: (2-aminoethyl)methanethiosulphonate; $ONOO^-$: peroxynitrite; PCMBS: *p*-chloromercuribenzene sulphonic acid; PHMPS: *p*-hydroxy-mercuric-phenylsulphonic acid; ROS: reactive oxygen species; SNAP: *N*-Acetyl-3-(nitrosothio)-D-valine; X+XO: xanthine+xanthineoxidase reaction.

In the following paragraphs we will review the cellular components that maintain the systolic redox milieu, and we will focus on how redox modulation of Ca^{2+} regulatory proteins affect cardiac excitation–contraction (e–c) coupling and function under physiological and pathophysiological conditions.

2. Intracellular redox potential and redox buffering system

The redox status of the intracellular milieu is controlled by a variety of redox active compounds. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered the major biologically relevant redox active molecules, whereas glutathione (together with the glutathione peroxidase/reductase system), nicotinamide-adenine dinucleotide redox couple ($NADH/NAD^+$), the thioredoxin (Trx) system and the enzymes superoxide dismutase and catalase constitute the key elements of cellular defense against redox injuries. Because levels of ROS and RNS can increase significantly after stimulation of specific signal transduction pathways or during certain pathological conditions of the heart (e.g. ischemia–reperfusion) the redox defense system is essential for the maintenance of cellular homeostasis.

2.1. Glutathione

In many mammalian cells, including cardiomyocytes, glutathione is considered the major cytosolic redox buffer. Under normal physiological conditions, glutathione is mainly reduced. The ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG; oxidized form) is >10 [6], however, under pathological conditions the GSH/GSSG ratio can decrease significantly [7]. The pentose phosphate pathway regulates the GSH/GSSG ratio by providing nicotinamide-adenine dinucleotide phosphate (NADPH), which is required for the reduction of GSSG to GSH by glutathione reductase. The maintenance of a high cytosolic GSH/GSSG ratio is a critical factor in antioxidant defense. GSH can directly scavenge free radicals including ROS or indirectly through the reaction catalyzed by glutathione peroxidase.

2.2. NADH/NAD$^+$

In contrast to glutathione, under normal aerobic conditions, cytosolic nicotinamide-adenine dinucleotides are mostly oxidized (i.e. present in form of NAD^+) with an $NADH/NAD^+$ ratio <0.05 [8]. This low ratio is the result of a low glycolytic flux and the ability of mitochondria to

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oxidize cytosolic NADH by the malate-aspartate and α-glycerol phosphate shuttles [9]. The ratio of $NADH/NAD^+$ fluctuates in response to changes in metabolism and can be interpreted as an indicator of the metabolic state of a cell or tissue. During ischemia, for example, cytosolic $NADH/NAD^+$ ratio can increase up to 30 times [10].

Cytosolic GSH/GSSG or $NADH/NAD^+$ ratios are usually estimated from whole cell extracts, providing only information about global changes in redox state of the cytosol. Due to specific localization of enzymes regulating cytosolic redox levels, it seems likely that redox regulation is highly compartmentalized with cytosolic subregions having variable redox states. For example, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme which regulates cytosolic $NADH/NAD^+$ levels, is associated with the SR membrane [12]. Thus, changes in activity of this enzyme may lead to more significant fluctuations of local $NADH/NAD^+$ levels in close proximity to SR bound proteins (such as the RyR and SERCA) than in the bulk cytosol.

2.3. Thioredoxin

The thioredoxin (Trx) system, which consists of Trx reductase and Trx peroxidase, is another important cellular system that regulates the redox state of the cytosol [13]. The Trx system plays an important role in cardioprotection against ROS by directly scavenging hydrogen peroxide and regulating signal transduction pathways [14,15].

2.4. Reactive oxygen species (ROS)

ROS are intermediates of the reduction of O_2 to water and include superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2). ROS play a critical role in many disorders of the cardiovascular system (e.g. [16–19]), such as ischemia–reperfusion injury, myocardial stunning, apoptosis, inflammation and arteriosclerosis. Potential sources of ROS include the mitochondrial electron transport chain, xanthineoxidase, cytochrome P450-based enzymes and NADH oxidase. Under certain conditions accumulation of ROS can trigger a burst of ROS production ("ROS-induced ROS release") by mitochondria that can lead to apoptotic cell death [20]. Superoxide dismutase (SOD), catalase and glutathione peroxidase are the main enzymatic components of cellular defense against oxidative damage. Furthermore, endogenous antioxidants including GSH, tocopherols, and ascorbic acid also participate in scavenging free radicals. Therefore, for normal functioning of the cell, the control of the balance between ROS production and elimination is pivotal. For example, a significant burst of ROS formation occurs during reperfusion of the ischemic heart [21–23], however, the exact source and mechanism of ROS formation during reoxygenation remains to be determined. In bovine cardiac myocytes a major source of $O_2^{-\cdot}$ production stems from a membrane associated NADH oxidase, an enzyme controlled by cytosolic $NADH/NAD^+$ levels and oxygen pressure (pO_2) [24]. Inhibition of NADH oxidase activity substantially suppresses ROS production [24,25]. Reperfusion after ischemia also leads to dramatic changes of Ca^{2+} homeostasis and e–c coupling, a process in which ROS play a crucial role [16]. Accumulation of ROS leads to cardiac Ca^{2+} overload [23,26,27] and it has been suggested that redox modulation of ion channel and pump activity is directly responsible for these pathological processes [1].

In addition to the well recognized role of ROS in acute ischemia/reperfusion-related Ca^{2+} overload, it is becoming increasingly evident that ROS also participate in signal transduction processes that control gene expression, cell growth and apoptosis. ROS production increases after receptor stimulation by specific hormones, growth factors and cytokines [17]. For example, in ventricular myocytes stimulation of α_1-adrenergic receptors activates NADPH oxidase-mediated ROS production and hypertrophic signaling pathways [28]. In contrast to the damaging effects of high concentrations of ROS, low levels of ROS participate in cellular signal transduction via modulation of SR Ca^{2+} release [29] or play a role in preconditioning-dependent myocardial protection against ischemia–reperfusion injury [30].

2.5. Nitric oxide (NO)

NO is generated by the family of enzymes of 3 isoforms of NO synthase (NOS): endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoenzyme. eNOS is the predominant isoform in the heart [31] where it is preferentially located in caveolae of the sarcolemma in the vicinity of β-adrenergic receptors and L-type Ca^{2+} channels [32]. nNOS has been found targeted to the SR and associated with the RyR Ca^{2+} release channel [32,33]. Co-localization of NOS with Ca^{2+} transport systems suggests an important role of NO in the regulation of cardiac e–c coupling. In the absence of sufficient amounts of substrate (L-arginine) or co-factors (tetrahydrobiopterin) NOS can produce $O_2^{-\cdot}$ [34,35]. Furthermore, because NO and $O_2^{-\cdot}$ combine spontaneously to form peroxynitrite ($ONOO^-$), NOS activity indirectly can contribute to $ONOO^-$ levels. Thus, by means of generating NO, $O_2^{-\cdot}$ and $ONOO^-$, NOS plays an important role in both ischemia–reperfusion injury and preconditioning [36]. In the normal heart the expression of iNOS is very low, however can significantly increase during pathological conditions such as cardiac infarction or inflammation [37], and a constitutive NOS can mediate negative inotropic effects of pro-inflammatory cytokines [38]. NOS has also been located to mitochondria in a variety of tissues, including the heart [39], where NO production can exert fast local modulatory effects on mitochondrial Ca^{2+} homeostasis, cell respiration, mitochondrial membrane potential and apoptosis.

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3. Redox modulation of cardiac ion channels and transporters

Contraction of cardiac myocytes is activated by an action potential-induced membrane depolarization and subsequent Ca^{2+} entry through dihydropyridine-sensitive L-type Ca^{2+} channels. This relatively small Ca^{2+} influx triggers the opening of SR Ca^{2+} release channels (RyRs), resulting in a massive Ca^{2+} release from the SR Ca^{2+} store. This mechanism which mediates the process of cardiac e–c coupling is known as Ca^{2+}-induced Ca^{2+} release (CICR) [40]. The global Ca^{2+} release is the result of spatial and temporal summation of many localized elementary Ca^{2+} release events, termed Ca^{2+} sparks [41]. Cardiac relaxation is due to termination of Ca^{2+} release with subsequent reuptake of cytosolic Ca^{2+} into the SR by SERCA and extrusion predominantly by Na^+–Ca^{2+} exchange (NCX) [42]. The relative contributions of NCX and SERCA to Ca^{2+} removal vary from species to species, whereas the sarcolemmal (plasmalemmal) Ca^{2+} ATPase (PMCA) plays a minor role [42]. In the following sections we review redox modulation of key Ca^{2+} channels and transporter relevant to cardiac e–c coupling.

3.1. Ryanodine receptor (RyR) Ca^{2+} release channel

Ca^{2+} release from the SR through RyR channels is essential for activation of cardiac and skeletal muscle contraction. Three isoforms of RyR have been identified. The RyR1 isoform is dominant in skeletal muscles, whereas the RyR2 represents the cardiac RyRs isoform. The RyR3 isoform is found only at low expression levels in certain skeletal muscle types. At the amino acid level, the three mammalian RyR isoforms share ~70% identity [43]. The RyR has multiple sites for regulation, including cytosolic and luminal sites for regulation by Ca^{2+}, and cytosolic sites for Mg^{2+}, ATP, calmodulin (CaM) and FK-506 binding proteins (FKBP) [43]. The RyR, a tetrameric complex (~2.5 MDa), contains up to 89 cysteine residues per monomer [44]. However, only a small number of hyperactive SH groups on the channel protein appear to have a redox-sensing function [2,45,46]. Reagents that specifically oxidize free SH groups and promote the formation of disulfide bonds within the RyR complex (2.2′-dithiodipyridine (DTDP) or 5.5′-dithio-bis(2-nitrobenzoic acid) (DTNB)) activate the channel. This effect is reversed by reagents that reduce disulfides to thiols (e.g. dithiothreitol (DTT)) [47–51]. The redox modulation of the RyR can also lead to alteration of the sensitivity of the channel to cytosolic Ca^{2+} and ATP [52,53]. Redox modification of cysteine residues alters the interaction of the RyR with triadin [46]. Triadin, a transmembrane ancillary protein, regulates the sensitivity of the RyR to luminal Ca^{2+} by stabilizing the binding of calsequestrin (an intra-SR Ca^{2+} binding protein) to the RyR [54]. Therefore, changes of the redox status of RyR SH groups may alter the sensitivity of the channel to both cytosolic and luminal Ca^{2+}. The intracellular redox buffer GSH/GSSG modulates RyR1 and RyR2 function: reducing conditions inhibit, whereas oxidizing conditions stimulate the activity of the channel [48,53,55,56]. The mechanism of redox modulation of RyR by GSH/GSSG involves an interaction with the CaM binding site of the channel. Oxidized glutathione (GSSG) stimulates RyR2 activity by decreasing the affinity of CaM to bind to the RyR [56]. Recent data from our laboratory demonstrated that the cytosolic redox buffer NADH modulates gating properties of the cardiac RyR [57–59]. NADH inhibited the current through RyR2 channels by a mechanism that required oxidation of NADH and was reversed by NAD^+ (Fig. 1A) and NADH significantly depressed local SR Ca^{2+} release (Ca^{2+} sparks) in isolated ventricular myocytes (Fig. 1B) [58]. Fig. 1C shows that Ca^{2+} spark frequency depends on the cytosolic NADH/NAD^+ ratio with reducing conditions (NADH/NAD^+ >1) significantly depressing Ca^{2+} sparks. At this moment, we do not know whether NADH directly interacts with cardiac RyR or with some ancillary proteins that remain associated to the RyR channel after incorporation into the bilayer. In support of this notion, skeletal muscle RyR1 contains an oxidoreductase-like domain that could function as a redox sensor [60].

RyR function is also affected by ROS. The majority of studies suggest that ROS effects are mediated via oxidation of SH groups of the RyR [1]. It has been shown that $O_2^{-\cdot}$ [4,58] and H_2O_2/$OH^\cdot$ [61–63] increase the open probability of cardiac and skeletal muscle RyRs and this effect is reversed by agents that reduce thiol groups, such as DTT. There is also evidence that after initial stimulation of channel activity, ROS can cause irreversible inactivation of RyRs [64]. The effects of ROS are likely to depend on concentration and the length of exposure. Experiments on single ventricular myocytes indicate that H_2O_2 causes Ca^{2+} overload as a result of activation of Ca^{2+} release from intracellular stores, but not from activation of voltage-dependent Ca^{2+} channels or NCX [27]. This observation is consistent with our previous work showing that $O_2^{-\cdot}$, generated from the xanthine/xanthineoxidase (X+XO) reaction, stimulates RyR2 channels incorporated into lipid bilayers (Fig. 2A) and local SR Ca^{2+} release in isolated ventricular myocytes (Fig. 2B) [58]. After an initial stimulation of release, prolonged exposure to $O_2^{-\cdot}$ caused depletion of SR Ca^{2+} as a result of inhibition of SERCA (see below). Qualitatively similar results were obtained with OH generated from *t*-butyl hydroperoxide (*t*-BHP). In ventricular cells, $OH^\cdot$ caused pro-arrhythmogenic spontaneous Ca^{2+} waves that were interspersed between electrically triggered $[Ca^{2+}]_i$ transients (Fig. 2C).

There are, however, significant experimental limitations of studying ROS-dependent modulation of intracellular signaling. Traditionally, exogenous ROS generating systems (such as the X+XO reaction) have been used, which cause a global increase in [ROS]. Due to high reactivity of free radicals, $OH^\cdot$ and $O_2^{-\cdot}$ have very limited diffusion distance in the range of only a few nanometers [65]. This fact

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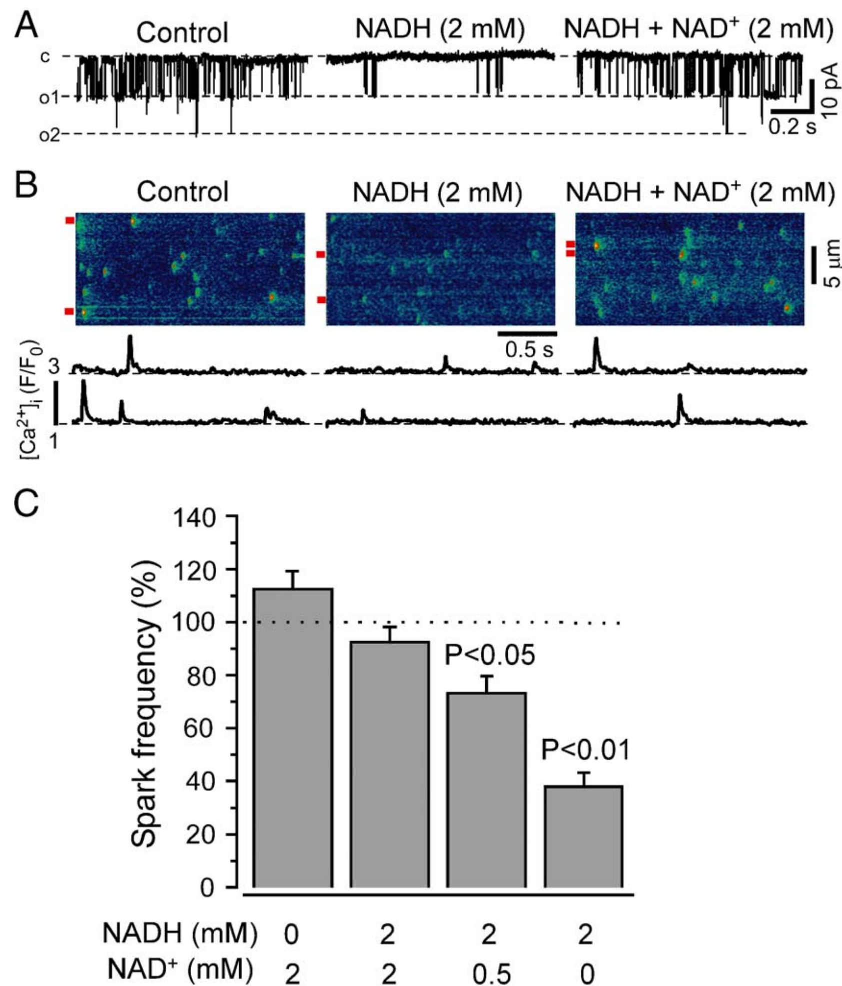

Fig. 1. NADH inhibits ryanodine receptor (RyR) activity and spontaneous Ca^{2+} sparks in ventricular myocytes. A, Effect of NADH and NAD^+ on single RyR channel activity (lipid bilayer measurements; holding potential = −20 mV; *cis*$[Ca^{2+}]$ = 3 μM). B, Effect of NADH and NAD^+ on Ca^{2+} spark activity in saponin-permeabilized ventricular myocytes. Confocal linescan images (fluo-3 fluorescence) and local F/F_0 profiles of Ca^{2+} release events. C, Average data of effects of different NADH/NAD^+ ratios on Ca^{2+} spark frequency. 100% = spark frequency in the absence of NADH and NAD^+. For details see [57,58]. Modified from [58].

suggests that ROS produced by endogenous systems would predominantly affect closely apposed targets, indicating that redox modulation is highly compartmentalized. In support of this notion both cardiac and skeletal RyRs were found to co-localize with an NADH oxidase, and locally generated $O_2^{-\cdot}$ can stimulate RyR channel activity [66,67].

The mechanisms of NO action on the gating properties of the RyR remain controversial. Activation of NOS generates NO free radicals (NO). NO can stimulate cGMP-mediated pathways by activating guanylate cyclase [68]. Furthermore, NO can freely oxidize to nitrosonium ion (NO^+) which can react with SH groups of cysteine residues, forming *S*-nitrosothiol [69]. The reaction of *S*-nitrosylation requires electron acceptors, such as O_2 or NAD^+. Nitrosylation of SH groups of the cardiac RyR by low molecular weight *S*-nitrosothiols, such as or *S*-nitrosoglutathione (GSNO) and *S*-nitrocysteine (CysNO), stimulates channel activity [44,70] (Fig. 3). SIN-1 (3-morpholinosydnonimine) which generates the NO-related species peroxynitrite activates the channel several fold through thiol oxidation [44]. On the other hand, NO produced endogenously by NOS located to the cardiac SR decreases the open probability of the RyR [71]. It seems that effects of NO or NO-related molecules depend on concentration and cytosolic environment. Low concentrations of NO donors have been shown to activate the RyR, whereas high concentrations cause inhibition of the channel [72]. The mechanisms of NO-mediated modulation of the skeletal RyR have been explored in more detail. It has been shown that from 50 free thiols per subunit of RyR1 only *S*-nitrosylation of one single cysteine, Cys3635, is responsible for activation of the channel by NO [73]. Substitution of Cys3635 abolishes NO effects, but does not prevent redox modulation of RyR by GSH/GSSG suggesting that more than one SH group of the RyR play a redox-sensing function. Cysteine residues could only be nitrosylated at low (i.e. physiological) pO_2, suggesting that this effect of NO was oxygen tension-dependent [73,74]. These results contrast the finding that NO failed to modulate RyR1 channel activity over a wide pO_2 range [75]. At the same time, *S*-nitrosothiols (CysNO or GSNO) modulate RyR

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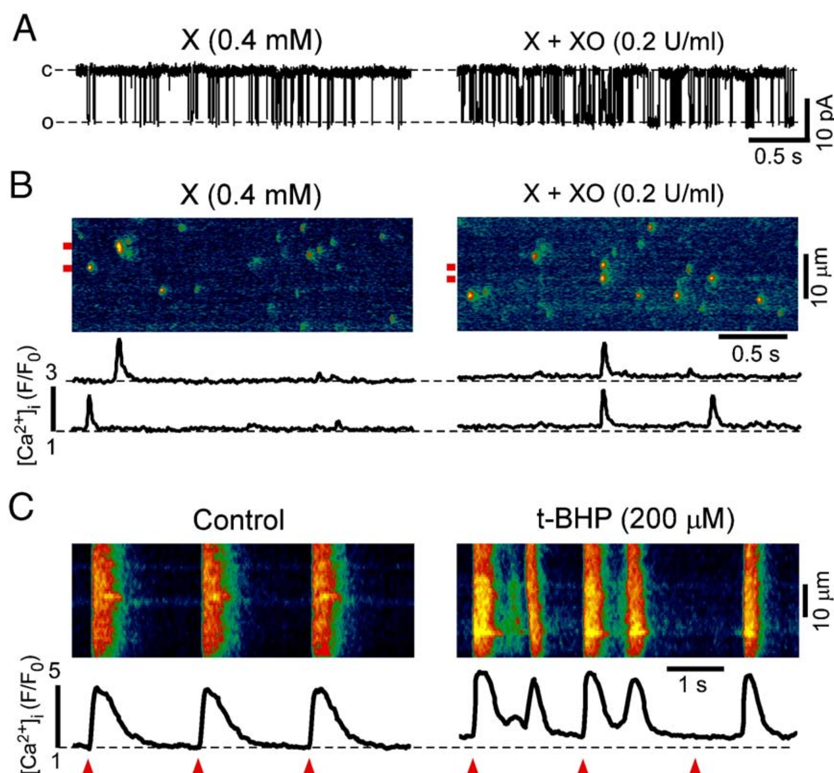

Fig. 2. ROS generated from xanthine/xanthineoxidase (X+XO) reaction and *t*-butyl hydroperoxide (*t*-BHP) stimulates single RyR activity, Ca^{2+} sparks and electrically evoked $[Ca^{2+}]_i$ transients in ventricular myocytes. A, RyR single channel recordings in the presence of 0.4 mM X and after addition of 0.2 U/ml XO. B, Ca^{2+} spark activity in the presence of 0.4 mM X and after addition of 0.2 U/ml XO in permeabilized ventricular myocytes. C, $[Ca^{2+}]_i$ transients in control conditions and in the presence of 200 μM *t*-BHP. Electrical stimulations are indicated by triangles. For details see [58]. Panels A and B modified from [58].

through nitrosylation of cysteine residues other than Cys3635 [76]. There is also evidence that nitroxyl (HNO), another byproduct of NO synthesis, can activate cardiac and skeletal RyR channels by a mechanism different from *S*-nitrosylation [77].

3.2. Inositol-1,4,5-trisphosphate receptor (IP_3R) channel

Similar to the RyR there are also three IP_3R isoforms referred to as type-1, -2 and -3, transcribed by three distinct genes [78]. Each IP_3R subtype has a different affinity for IP_3 and a specific tissue expression pattern. The role of IP_3R in mobilization of Ca^{2+} from intracellular stores in non-excitable cells is well established [79]. In contrast, in the heart the main pathway of Ca^{2+} release occurs through RyRs, and IP_3Rs (type-2) are expressed at 1–2 orders of magnitude lower density than RyRs [80]. Thus, the role of IP_3 in cardiac e–c coupling in the adult mammalian heart has remained highly controversial [42,81], however there is growing evidence that Ca^{2+} release via IP_3R modulates e–c coupling and contributes to arrhythmias in atrial tissue [82,83].

Evidence for redox modulation of the IP_3R stems only from non-muscle tissue. In HeLa cells the thiol reagent thimerosal stimulates Ca^{2+} release from intracellular stores by sensitizing the IP_3R to basal IP_3 levels [84]. This effect of thimerosal was reversed by DTT, suggesting that redox modification of SH groups of the IP_3R protein might be involved. In permeabilized hepatocytes oxidized glutathione (GSSG) stimulates the IP_3R by increasing the binding affinity of IP_3 to the receptor [85]. Furthermore, it has been shown recently that the glycolytic enzyme GAPDH is associated with the IP_3R suggesting that changes in the formation of NADH may also regulate Ca^{2+} signaling through IP_3R [86]. In cerebellum, for example, NADH increased IP_3-mediated Ca^{2+} flux through the purified IP_3R [87].

The IP_3R can also be modulated by ROS. *t*-BHP increased IP_3R-mediated Ca^{2+} release in hepatocytes. This effect could be mimicked by the addition of GSSG and reversed by pretreatment with DTT [88]. $O_2^{-\cdot}$ generated from the X+XO reaction stimulated Ca^{2+} release through the IP_3R in smooth muscle cells [89]. The effect of $O_2^{-\cdot}$ was not seen when a nonhydrolyzable analogue of IP_3 was used to induce Ca^{2+} release. This fact suggests that $O_2^{-\cdot}$ inhibits the degradation processes of IP_3, rather than directly stimulating the IP_3R.

In cardiac myocytes IP_3 and NO signaling pathways are interconnected and affect Ca^{2+} homeostasis and e–c coupling [90], however it remains to be determined whether and to what extent redox processes are involved.

3.3. Voltage-dependent L-type Ca^{2+} channel

During e–c coupling membrane depolarization by an action potential leads to Ca^{2+} influx through voltage-gated

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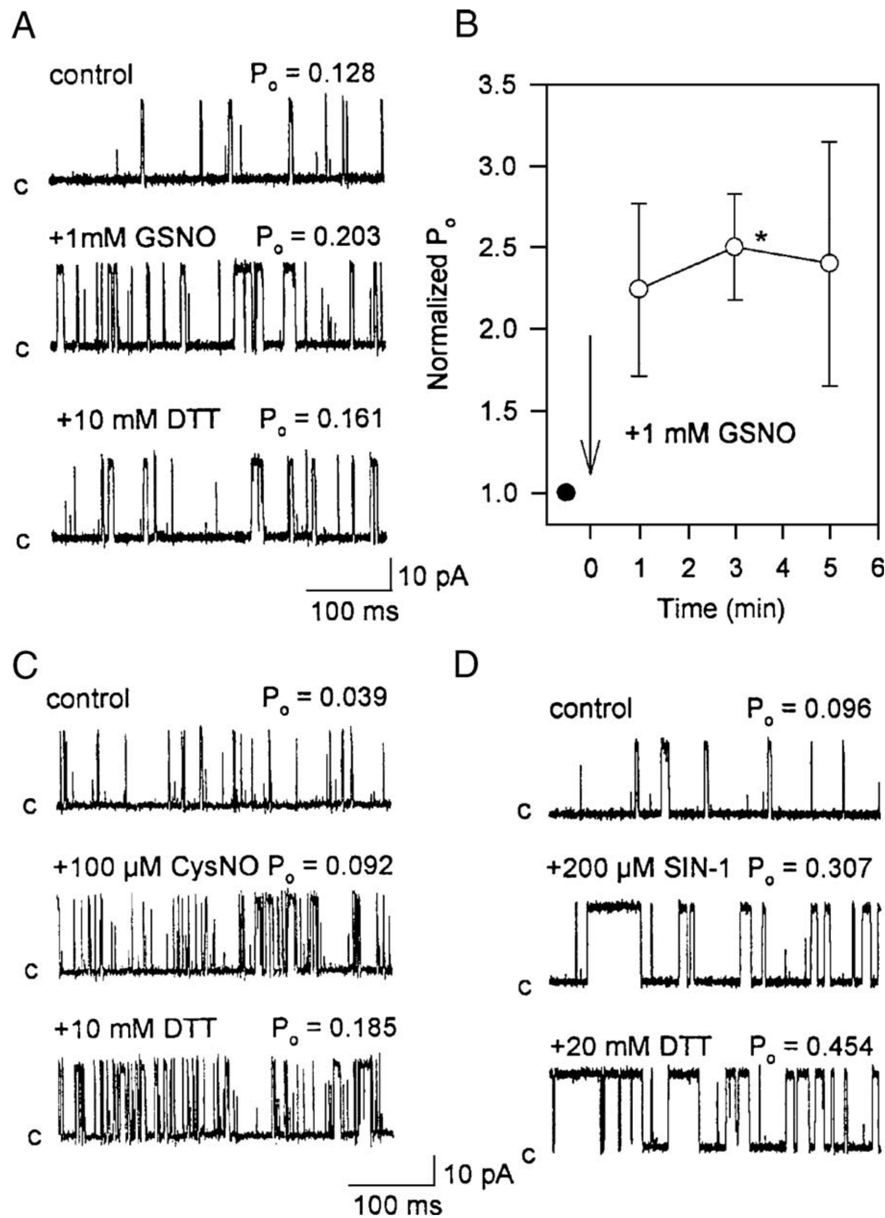

Fig. 3. Effects of GSNO (A, B), CysNO (C), and SIN-1 (D) on cardiac RyR activity. Reprinted with permission from Xu, L. et al., SCIENCE 279: 234–237 (9 January 1998). Copyright 1998 AAAS.

dihydropyridine-sensitive L-type Ca^{2+} channels which is followed by CICR from the SR. L-type Ca^{2+} channel contains 5 subunits (α_1, α_2, β, γ and δ). The pore-forming subunit α_{1C} of the cardiac L-type Ca^{2+} channel contain more than 10 cysteine residues [91] which can potentially undergo redox modification. Indeed, thiol oxidizing agents (e.g. thimerosal) irreversibly decreased current through the human α_{1C} subunit (reversed by DTT) [92] and rabbit cardiac Ca^{2+} channels [93], both expressed in HEK293 cells. In isolated guinea pig ventricular myocytes DTT did not affect Ca^{2+} current (I_{Ca}) directly, but reversed current inhibition by thiol oxidants [94]. Furthermore, ROS presumably acting as oxidants of SH groups, also decreased cardiac L-type I_{Ca} [95,96]. These results, however, are controversial because in ferret ventricular myocytes agents that oxidize SH groups (DTNB) caused stimulation of I_{Ca}, whereas GSH and DTT which reduce disulfide bonds inhibited the current [97].

NO modulates cardiac L-type I_{Ca} by two different mechanisms, consisting of a direct redox-dependent stimulation and an indirect cGMP-dependent inhibition [97]. It has been shown that *S*-nitrosothiols (CysNO and GSNO) increased the amplitude of I_{Ca} and DTT partially reversed this effect in ferret ventricular myocytes [97]. NO-mediated stimulation of I_{Ca} was the result of *S*-nitrosylation of extracellular SH groups of the L-type Ca^{2+} channel, but was independent of the activity of kinases, phosphatases or SR Ca^{2+} release. In contrast to these results, *S*-nitrosothiols have been reported to inhibit Ba^{2+}

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currents through rabbit cardiac Ca^{2+} channel subunits expressed in HEK293 cells [93].

3.4. SR Ca^{2+} ATPase (SERCA)

Relaxation of cardiac muscle relies on Ca^{2+} sequestration by SERCA into the SR. Three SERCA isoforms have been identified in muscle cells. SERCA1 isoform is predominant in fast skeletal muscle, whereas SERCA2a is expressed in slow skeletal and cardiac muscle. The SERCA2b isoform is the main isoform in smooth muscle, while SERCA3 is expressed in non-muscle cells. Redox agents are known to modulate SERCA. SERCA contains 25 cysteine residues, but only 1 or 2 are essential for enzyme action [98]. Thus, the redox state of these catalytic SH groups can determine the activity of the SR Ca^{2+} ATPase. In contrast to the RyR, reagents that oxidize thiols (e.g. DTNB) inhibit pump activity, whereas reducing agents (e.g. DTT and GSH) protect SERCA from this inhibition [99,100]. The activity of SERCA is also sensitive to oxidative stress. $O_2^{-\cdot}$ and $H_2O_2/OH^{\cdot}$ inhibit Ca^{2+} uptake into the SR [5,58,100–103]. Because Ca^{2+} transport into the SR is tightly coupled to hydrolysis of ATP, inhibition of ATPase activity will ultimately decrease the Ca^{2+} pumping rate. $H_2O_2/OH^{\cdot}$ inactivates cardiac and skeletal muscle SERCA by directly interfering with the ATP binding site [103]. Furthermore, ROS cause inhibition of SERCA function by uncoupling Ca^{2+} uptake activity from ATP hydrolysis [5]. Additionally, ROS may inhibit the activity of membrane-bound enzymes by peroxidation of membrane phospholipids [100,101]. Despite the fact that all SERCA isoforms share 90% in amino acid homology, they exhibit different sensitivities to ROS. For example, cardiac SERCA2a has been found to be more sensitive to $H_2O_2/OH^{\cdot}$ than to $O_2^{-\cdot}$ [5] and SERCA3 isoform is more resistant than SERCA2b to peroxides [102].

NO does not appear to affect SERCA Ca^{2+} pump activity by *S*-nitrosylation of cysteine residues directly [70,74,104], however $ONOO^-$ was found to increase activity of SERCA2 [104]. This stimulation was dependent on GSH level and formation of *S*-glutathiolation adducts with cysteine residues of SERCA. In addition, mutation of residue Cys674 to serine abolished these effects. During pathological conditions that are associated with increases in ROS production, this mechanism of NO signaling is likely to be abolished as a result of irreversible oxidation of reactive SH groups (mainly Cys674) [104].

3.5. Plasmalemmal Ca^{2+} ATPase (PMCA)

PMCA, like SERCA, is a member of P-type ATPases. Due to the slow pumping rate, however, PMCA activity is considered to be less relevant for beat-to-beat Ca^{2+} extrusion and cardiac relaxation [42]. ROS can substantially inhibit both Ca^{2+} transport and ATPase activity of PMCA [105,106]. These effects of ROS occur through oxidation of SH groups and peroxidation of membrane phospholipids. Furthermore, $ONOO^-$ irreversibly inhibited PMCA Ca^{2+} pump in rat brain synaptosomes [107], suggesting that a similar effect may occur in cardiac tissue.

3.6. Na^+–Ca^{2+} exchange (NCX)

Cardiac myocytes express sarcolemmal NCX protein at high levels, consistent with the important role of NCX for maintaining Ca^{2+} homeostasis. Using the transmembrane Na^+ gradient, NCX typically pumps Ca^{2+} out of the cell electrogenically (Na^+:Ca^{2+}=3:1), however membrane depolarization or high $[Na^+]_i$ can cause Ca^{2+} entry via reverse mode action of NCX. Cardiac NCX (encoded by the gene NCX1) consists of nine transmembrane domains, and intramolecular disulfide bonds between cysteine residues of different domains have been implicated to be functionally relevant [108]. The activity of the cardiac NCX is sensitive to redox modification by ROS [109,110]. $O_2^{-\cdot}$ generated from the X+XO reaction (but not by H_2O_2) substantially enhanced NCX-mediated Ca^{2+} fluxes [109]. Shown by a different study, NCX could also be stimulated by ROS generated from both H_2O_2 and X+XO, but was inhibited by the strong oxidant HOCl [110]. Redox modification of NCX by the combination of $FeSO_4$ and DTT removed Na^+-dependent inactivation of NCX and activated the exchanger [111]. These effects were not dependent on a rearrangement of disulfide bonds between cysteine residues. In isolated ventricular myocytes, ROS generated from H_2O_2 and X+XO augmented NCX activity [112]. Because significant bursts of ROS generation occur during reperfusion of the ischemic heart, changes of NCX activity are likely to play an important role in ischemia-related Ca^{2+} overload. This is consistent with the observation that hypoxia profoundly inhibits NCX activity in guinea pig ventricular myocytes, whereas reoxygenation reactivates the exchanger [113]. Therefore, stimulation of NXC by ROS during reperfusion, when $[Na^+]_i$ is elevated, may promote the reverse mode of NCX and lead to Ca^{2+} overload.

3.7. Other ion channels and transporters in the heart

Different types of cardiac K^+ channels are sensitive to redox modulation. GSSG or DTNB inhibit the transient outward current (I_{to}) through oxidation of SH groups on the channel protein in ventricular myocytes [114]. ROS can cause suppression of outward K^+ currents [115], and inwardly rectifying K^+ current [116]. It has been suggested that modulation of K^+ channels by ROS results in early afterdepolarizations and arrhythmias [117]. In contrast to inhibition of K^+ channels that contribute to action potential configuration under normal physiological conditions, ROS stimulate the ATP-sensitive K^+ channel that usually becomes activated under pathological conditions of the heart involving ATP depletion [96,118]. Furthermore, redox-dependent modulation of activity of Na^+–K^+ ATPase [119] and Na^+–H^+ [120] exchange has been reported.

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4. Conclusion

Redox modulation of biochemical pathways and signaling cascades extends to Ca^{2+} channels and transporters, and underlies a number of cardiac dysfunctions and pathologies. There is a tendency that oxidation of ion transport systems that participate in cardiac e–c coupling can potentially lead to Ca^{2+} overload, whereas reduction tends to have an opposite effect. For example, ROS increase $[Ca^{2+}]_i$ by stimulation of SR Ca^{2+} release via RyRs (or IP_3Rs) and activation Ca^{2+} entry via the reverse mode of NCX. On the other hand, oxidative stress decreases of Ca^{2+} sequestration and extrusion by inhibiting SERCA and PMCA. All these ROS effects can take place during reperfusion of the ischemic heart and are likely to be responsible for abnormalities of Ca^{2+} homeostasis and e–c coupling.

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