

Invited review

Cross-talk between calcium and reactive oxygen species signalingYuan YAN, Chao-liang WEI, Wan-rui ZHANG, He-ping CHENG, Jie LIU¹*Laboratory of Calcium Signaling, Institute of Molecular Medicine, Peking University, Beijing 100871, China***Key words**

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Abstract

Calcium (Ca²⁺) and reactive oxygen species (ROS) constitute the most important intracellular signaling molecules participating in the regulation and integration of diverse cellular functions. Here we briefly review cross-talk between the two prominent signaling systems that finely tune the homeostasis and integrate functionality of Ca²⁺ and ROS in different types of cells. Ca²⁺ modulates ROS homeostasis by regulating ROS generation and annihilation mechanisms in both the mitochondria and the cytosol. Reciprocal redox regulation of Ca²⁺ homeostasis occurs in different physiological and pathological processes, by modulating components of the Ca²⁺ signaling toolkit and altering characteristics of local and global Ca²⁺ signals. Functionally, interactions between Ca²⁺ and ROS signaling systems can be both stimulatory and inhibitory, depending on the type of target proteins, the ROS species, the dose, duration of exposure, and the cell contexts. Such extensive and complex cross-talk might enhance signaling coordination and integration, whereas abnormalities in either system might propagate into the other system and undermine the stability of both systems.

Introduction

The calcium (Ca²⁺) ion, as a ubiquitous intracellular messenger, regulates many different cellular functions, including contraction, secretion, metabolism, gene expression, cell survival and cell death^[1]. Likewise, reactive oxygen species (ROS) such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) are widely involved in physiological and pathophysiological processes through oxidizing proteins, lipids and polynucleotides^[2,3]. Recent studies have underscored the notion that the Ca²⁺ and ROS signaling systems are intimately integrated such that Ca²⁺-dependent regulation of components of ROS homeostasis might influence intracellular redox balance, and *vice versa*. On one hand, a number of ROS-generating and antioxidant systems of living cells have been shown to be Ca²⁺-dependent^[4,5]. Conversely, regulation of Ca²⁺ signals can be redox-dependent. The incredible versatile Ca²⁺ signals, depending on an extensive Ca²⁺ signaling toolkit, can act in various contexts of space, time and amplitude^[1,6]. Redox modulation of components of the Ca²⁺ signaling toolkit occurs in different physiological and pathological processes, resulting in altered amplitude and spatiotemporal characteristics of Ca²⁺ signals.

In this brief review, we discuss the specific mechanisms underlying the interaction and integration of these two powerful intracellular signaling systems in different types of cells.

Ca²⁺ modulation of ROS homeostasis

ROS play an important role in physiological cellular functions by activating several enzymatic cascades and transcription factors^[7]. Excessive ROS signals, however, are detrimental, causing Ca²⁺ overload, mitochondrial depolarization, cytochrome c release, lipid peroxidation, transcription factor activation and DNA damage, and lead to apoptotic and non-apoptotic cell death. As such, oxidative stress is increasingly recognized as a causative factor in the development of a diverse array of diseases, including neurodegenerative diseases, malignant diseases, diabetes mellitus, atherosclerosis, and ischemia/reperfusion injury^[7]. The intracellular redox state reflects the dynamic balance between ROS production and the antioxidant capacity of the cell. Increasing evidence indicates that intracellular Ca²⁺ modulates both ROS generation and ROS clearance processes and thereby shifts the redox state toward either a more oxidized or reduced direction in a context-sensitive manner.

Ca²⁺-induced ROS production There are many intracellular ROS generation sites (Figure 1). Of them, the electron transport chain resides on the mitochondria and there are plenty of extramitochondrial enzymes in the plasma membrane or in the cytosol, such as cell-surface NADPH-oxidase, peroxisomes, cytochrome P450, xanthine oxidase, cyclooxygenase, and lipoxygenase^[8,9]. Mitochondria provide the main source of physiological ROS production, with 1%–2% of total electrons flowing through the respiratory chain leaking to produce ROS^[10]. The primal ROS made by electron transport chains is O₂⁻, which is changed to H₂O₂ either by spontaneous dismutation or catalyzed by superoxide dismutase (SOD). One important function of mitochondrial Ca²⁺ is to stimulate the tricarboxylic acid (TCA) cycle^[11] and oxidative phosphorylation^[12–15]. Specifically, 3 dehydrogenases of the TCA cycle (pyruvate dehydrogenase, isocitrate dehydrogenase and oxoglutarate dehydrogenase)^[11], the ATP synthase (complex V)^[14], and the adenine nucleotide translocase^[12] are all activated by Ca²⁺. Hence, Ca²⁺ might increase ROS generation by enhancing metabolism. During this process, more electrons leak from the respiratory chain while more O₂ is consumed to produce ATP. To this end, previous studies have shown a positive correlation between mitochondrial ROS generation and the basal metabolic rate^[16,17]. Interestingly, Ca²⁺ can also enhance ROS produc-

tion when complexes of the electron transport chain are inhibited. As shown by *in vitro* experiments, Ca²⁺ stimulates ROS production in isolated rat heart mitochondria in the presence of antimycin A (complex III inhibitor)^[18]. Similar observations have been made with rotenone (complex I inhibitor) treatment of brain mitochondria^[19]. This phenomenon appears to be tissue-specific, because addition of Ca²⁺ to brain mitochondria in the presence of antimycin A does not stimulate ROS generation^[19].

The underlying mechanism for Ca²⁺-induced mitochondrial ROS generation is not fully understood. Cadenas and Boveris proposed that mitochondria depolarization is responsible for the Ca²⁺ effects^[18], whereas others have attributed the Ca²⁺ effects to the alteration of mitochondrial membrane structure^[19]. Studies on isolated mitochondria^[4,20–22] have demonstrated that high concentration of mitochondrial Ca²⁺ ([Ca²⁺]_m) triggers mitochondrial permeability transition pore (mPTP) opening and enhances ROS production, but the cascade of events linking mPTP opening to ROS generation remains elusive. The Ca²⁺-induced mPTP opening can be inhibited by antioxidants such as MCI-186^[23] or catalase^[24]. Furthermore, nearly 100 different proteins are lost from the mitochondrial inner membrane, including cytochrome c, glutathione (GSH) and other matrix solutes during mPTP opening. In principle, any of these molecules could enhance ROS

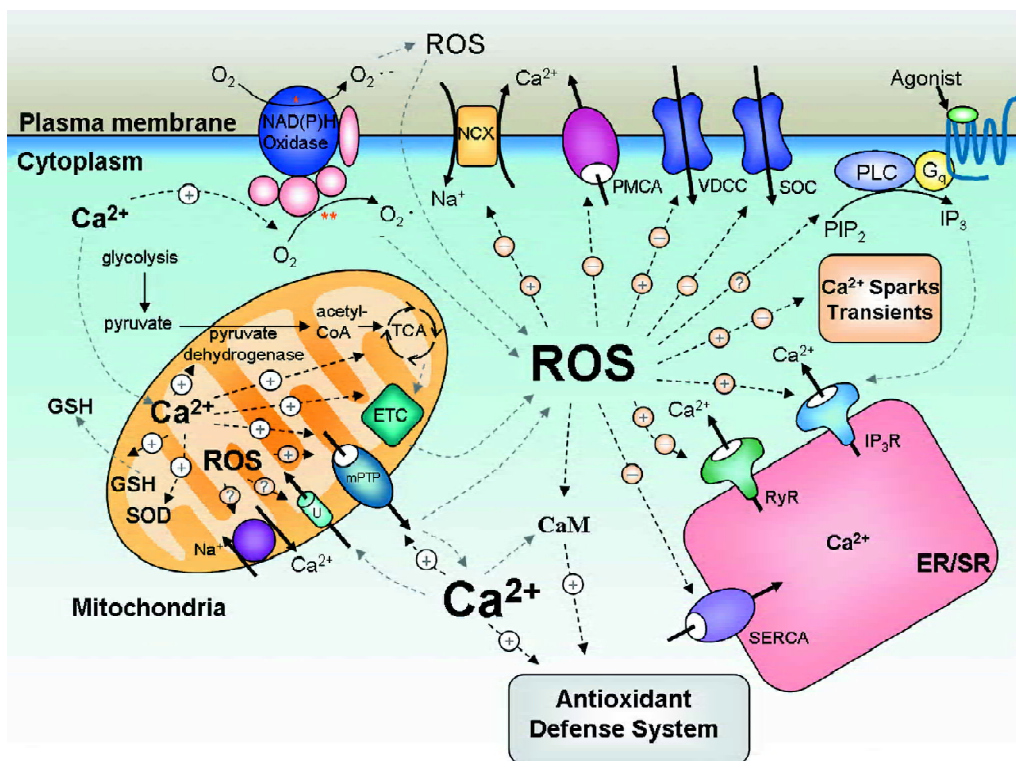


Figure 1. Cross-talk between Ca²⁺ and ROS signaling. Ca²⁺ modulates ROS generation and annihilation systems in the mitochondria and the cytosol. Meanwhile, components of Ca²⁺ signaling toolkit are regulated by ROS, so as to alter local and global Ca²⁺ signals in physiological and pathological processes. SOC, store-operated Ca²⁺ channel; PLC, phospholipase C; PIP₂, phosphatidylinositol-4,5-bisphosphate; TCA, tricarboxylic acid; ETC, electron transport chain; U, uniporter.

generation.

In addition to the regulation of mitochondrial ROS production, Ca^{2+} regulates multiple extramitochondrial ROS-generating enzymes both in physiological and pathological processes. Cell-surface NADPH oxidases, with rapid kinetics of activation and inactivation, are the most important multienzyme complexes in the generation of ROS involved in receptor-mediated signaling cascades^[8]. The best studied among them is the phagocyte NADPH-oxidase, which consists of a dimer of transmembrane subunits, gp91^{phox} and p22^{phox}, and three cytosolic subunits, p67^{phox}, p47^{phox}, and rac2. An additional component, p40^{phox}, is also associated with oxidase, but its functional role is unclear^[25]. Activity of neutrophil oxidases, including NADPH-oxidase, is Ca^{2+} -dependent^[26]. Buffering intracellular or extracellular Ca^{2+} decreases generation of oxygen metabolites in human neutrophils^[27]. NAD(P)H oxidase and its homologs are present in a variety of nonphagocytic cells including smooth muscle cells, chondrocytes, kidney epithelial cells, endothelial cells, prostate cancer cells^[21], and spermatocytes^[28]. In response to elevations of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), NADPH oxidase 5 (NOX5), a homolog of the gp91^{phox} subunit of the phagocyte NADPH oxidase, generates large amounts of superoxide^[28], which attributes to conformation change of NOX5 induced by Ca^{2+} ^[29]. It has been shown^[30,31] that activities of other ROS-generating enzymes are regulated by $[\text{Ca}^{2+}]_c$ directly or indirectly.

Ca^{2+} regulation of antioxidant defense system To counteract the damaging potential of ROS, cells use the antioxidant defense system, which involves both enzymatic and nonenzymatic oxidant defense mechanisms. Ca^{2+} can directly activate antioxidant enzymes, such as plant catalase and GSH reductase, increase the level of SOD in animal cells^[5], and induce mitochondrial GSH release early in Ca^{2+} -induced mPTP opening^[4]. Alternatively, calmodulin (CaM), a ubiquitous Ca^{2+} -binding protein, interacts with antioxidant enzymes involved in ROS homeostasis. CaM binds to and activates some plant catalases in the presence of Ca^{2+} , and down-regulates H_2O_2 levels^[32]. Collectively, these studies indicate that Ca^{2+} plays dual roles in regulating ROS homeostasis. The net Ca^{2+} effects on ROS generation and annihilation appear to be tissue-specific and context-sensitive, and, within a given cell, are differentially regulated in local subcellular compartments.

ROS regulation of Ca^{2+} signaling

The Ca^{2+} signaling system comprises hundreds and up to thousands of protein players that are involved in virtually

every aspect of cell biology and physiology. Any influence on the Ca^{2+} signaling toolkit might change the spatiotemporal profile of local and global Ca^{2+} signals, contributing to the efficiency, specificity and complexity of Ca^{2+} signal transduction. In this section we briefly discuss how ROS modify key Ca^{2+} signaling proteins and reshape local and global Ca^{2+} signal amplitudes and kinetics (Figure 1).

Voltage-dependent Ca^{2+} channels Ca^{2+} entry into excitable cells through voltage-dependent Ca^{2+} channels (VDCCs) is essential for membrane electrical activity and intracellular signal transduction. Many studies have focused on ROS modulation of VDCC activity. H_2O_2 has been shown to accelerate the overall channel opening process in neuronal P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes^[8]. Studies in whole-cell-clamped guinea pig ventricular myocytes have shown that exogenous ROS suppresses L-type Ca^{2+} current^[33]. Similarly, sulphhydryl-oxidating agents, 2,2-dithiodipyridine and thimerosal, also inhibit the activity of rabbit smooth muscle L-type Ca^{2+} channels expressed in CHO cells^[34], and it was found that free SH groups of L-type Ca^{2+} channels are essential for ROS modulation. Although the effects of H_2O_2 and other ROS on single DHPR channel activity have not been reported; previous studies indicate a ROS-induced decrease in this current of skeletal muscle^[31]. In contrast, it has also been reported that H_2O_2 exerts no significant effect on L-type Ca^{2+} current in pancreatic β -cells^[35]. In *Arabidopsis* guard cells, Pei and colleagues^[36] identified a hyperpolarization-dependent Ca^{2+} -permeable cation channel that is activated by H_2O_2 .

Intracellular Ca^{2+} release channels The release of Ca^{2+} from the endo/sarcoplasmic reticulum (ER/SR) mediated by ryanodine receptors (RyR) and 1,4,5-inositol-triphosphate receptors (IP₃R) is a primary Ca^{2+} signaling event. An RyR or IP₃R channel is a homotetramer with each subunit containing many free cysteine residues that are susceptible to redox reaction by ROS. For instance, each of the four homologous 560 kDa RyR₁ proteins contains approximately 50 free cysteine residues^[37], and approximately 21 free cysteines per subunit of RyR₂^[38]. Changes in the redox state of RyR and IP₃R would affect their activities. There are three types of RyR expressed in mammalian cells, known as RyR₁, RyR₂, and RyR₃. RyR₁ is the dominant isoform in skeletal muscle, RyR₂ is found in high levels in cardiac muscle, and RyR₃ is expressed at relatively low levels in many tissues including diaphragm and brain^[39]. RyR₁ channels, *in vitro*, were markedly activated by 100 $\mu\text{mol/L}$ and 1 mmol/L H_2O_2 under redox potential clamp conditions^[40]; were inhibited by 10 mmol/L H_2O_2 ^[41]. Moreover, 3–5 mmol/L H_2O_2 directly modified the gating of sheep cardiac RyR₂, resulting in an increase in chan-

nel open probability without affecting the conductance^[33]. Similarly, H₂O₂ enhances Ca²⁺ release from SR in isolated ventricular myocytes. This effect is more prominent in cells previously dialyzed with low concentration thiol reductants, GSH (2 mmol/L) or dithiothreitol (DTT; 0.5 mmol/L)^[42]. It is noteworthy that high concentration GSH (10 mmol/L) or DTT (2 mmol/L) itself strongly inhibits Ca²⁺ release in cardiomyocytes^[42]. In neurons, activation of RyR₃ by ROS might modify Ca²⁺-dependent long-term potentiation and long-term depression^[37]. In the case of IP₃R, it has been reported that O₂⁻ enhances IP₃-induced Ca²⁺ release from fractionated vascular smooth muscle SR^[43], and oxidized GSH induces Ca²⁺ release from IP₃R in intact hepatocytes^[44]. Moreover, the data reported by Hu *et al*^[45] demonstrated that exogenous NADPH (substrate of NADPH oxidase) or H₂O₂ increases the sensitivity of intracellular Ca²⁺ stores to IP₃ in human endothelial cells. Despite these advances, it remains to be convincingly demonstrated whether endogenous ROS can appropriately modify RyR and IP₃R activity in intact cells.

Ca²⁺ pumps and Na⁺/Ca²⁺ exchanger Both the plasma membrane Ca²⁺-ATPases (PMCA) and the ER/SR Ca²⁺-ATPases (SERCA), as well as Na⁺/Ca²⁺ exchangers (NCX), are sensitive to ROS regulation. ROS can effectively inhibit Ca²⁺ transport by SERCA in smooth muscle cells^[2] and depress cardiac sarcolemmal Ca²⁺-ATPase^[46]. SERCA is more sensitive to ROS than PMCA is. For example, H₂O₂ and O₂⁻ can completely uncouple the hydrolytic reaction of PMCA and inhibit the hydrolytic reaction of SERCA^[33]. Both stimulating and inhibiting regulation of ROS on NCX have been reported in isolated sarcolemmal vesicles and in intact cells. It has been proposed that H₂O₂ generated from the xanthine/xanthine oxidase system (X/XO) enhances NCX activity in ventricular myocytes, causing Ca²⁺ overload and triggering arrhythmia during reperfusion, because of the NCX pathological inverted running^[47]. Similar results were obtained in sarcolemmal vesicles from bovine heart^[48,49]. In contrast, oxidants from hypoxanthine/xanthine oxidase depress NCX activity in guinea pig ventricular myocytes under voltage-clamp conditions^[50]. The exchanger activity is also inhibited by the oxidizing agent HOCl^[48]. Although mitochondrial NCX and Ca²⁺ uniporter have been reported to participate in mitochondrial Ca²⁺ regulation^[51], it is not yet clear how they are modulated by intramitochondrial ROS.

Other components of the Ca²⁺ signaling system that are modulated by ROS include store-operated Ca²⁺ channel^[51], K_{Ca} channel^[33,52], and CaM^[8]. Taken together, ROS as intracellular signaling molecules might directly and indirectly modify components of Ca²⁺ signaling pathways, thus altering Ca²⁺ homeostasis and reshaping local and global Ca²⁺

signals.

Global Ca²⁺ signaling It has been widely accepted that exogenous ROS could induce dynamic changes in [Ca²⁺]_c in a variety types of cells^[54-59]. This effect might be due to mobilization of intracellular Ca²⁺ stores and to influx of extracellular Ca²⁺. As an important feature of the cross-regulation between ROS and Ca²⁺, the ROS effect on Ca²⁺ signaling can vary from stimulative to repressive, depending on the type of oxidants, their concentrations, and duration of exposure. When treated with 100 μmol/L H₂O₂, [Ca²⁺]_c of rat cardiomyocytes increased markedly, and continued to rise after washout, whereas 1 μmol/L H₂O₂ had no effect on [Ca²⁺]_c^[58]. At an even higher dose, 1 mmol/L H₂O₂ elicits biphasic response in cardiac myocytes, a transient augmentation of Ca²⁺-induced Ca²⁺ release followed by a suppression of [Ca²⁺]_c transient after 5 min exposure. The biphasic nature could be explained by a possible ER/SR depletion due to a combination of release enhancement and SERCA inhibition (Figure 1). Conversely, reducing agents such as GSH and DTT attenuate [Ca²⁺]_c transients^[3]. The effect of ROS on Ca²⁺ signaling is also tissue specific. For instance, it had been shown that H₂O₂ (100–300 μmol/L) activates contraction in skinned skeletal muscle fibers without producing an increase in [Ca²⁺]_c^[60]. Under pathological conditions, such as hypoxia and ischemia/reperfusion injury, mitochondria dysfunction results in ROS increase that mediates the following cytosolic Ca²⁺ overload^[4,59] by triggering Ca²⁺ release from the ER through RyR^[59] or from the external through PMCA^[4].

Local Ca²⁺ signaling Ca²⁺ sparks^[61] constitute the elementary Ca²⁺ releasing events and play an important role in local control of Ca²⁺ signaling in many types of cells. As is the case with ROS regulation of global Ca²⁺ signaling, ROS modulation of Ca²⁺ sparks occurs in a ROS species- and tissue-specific fashion. For example, O₂⁻ generated from X/XO elicits a slowly developing decrease of Ca²⁺ spark frequency down to 56% of control in permeabilized rat ventricular myocytes^[62]. In contrast, mitochondria-derived ROS, generated from diazoxide (an ATP-sensitive K⁺ channel opener)-induced mitochondrial depolarization, elevates Ca²⁺ spark frequency and enhances the coupling of sparks to Ca²⁺-sensitive K⁺ channels in smooth muscle cells^[63]. In permeabilized rat skeletal muscle fibers, 50 μmol/L H₂O₂ was also found to increase Ca²⁺ spark frequency^[64]. More direct evidence is needed to confirm the regulation of local and global Ca²⁺ signals by mitochondria-derived ROS in physiological and pathological conditions.

Concluding remarks

Cross-talk between Ca²⁺ and ROS signaling systems oc-

curs at multiple levels in different subcellular compartments (eg, the plasma membrane, the cytosol and mitochondria), and involves a constellation of molecular players (Figure 1). The reciprocal interactions between Ca²⁺ and ROS signaling systems can be both stimulatory and inhibitory, depending on the type of target proteins, the ROS species, the dose, the time history, and the cell contexts. Both ROS generation and clearance as well as Ca²⁺ signaling are subject to tight local regulation, therefore future study should unravel endogenous high local or compartmentalized ROS (eg, inside the mitochondrial matrix, ER/SR lumen or nucleoplasm) interacting with Ca²⁺ signaling molecules, and vice versa. Such cross-talk provides not only a fine-tuning mechanism for homeostatic regulation of either system, but also a coupling mechanism for signaling integration in the regulation of physiological functions. Under pathophysiological conditions, however, abnormalities in either signaling system could propagate into the other system, and feedback reinforcement could cause instabilities in both systems. We eagerly await future investigations to enlighten us on the cell logic behind the complex bi-directional interactions of the two signaling systems.

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