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Comprehensive Invited Review

Crosstalk Between Calcium and Redox Signaling: From Molecular Mechanisms to Health Implications

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ABSTRACT

Studies done many years ago established unequivocally the key role of calcium as a universal second messenger. In contrast, the second messenger roles of reactive oxygen and nitrogen species have emerged only recently. Therefore, their contributions to physiological cell signaling pathways have not yet become universally accepted, and many biological researchers still regard them only as cellular noxious agents. Furthermore, it is becoming increasingly apparent that there are significant interactions between calcium and redox species, and that these interactions modify a variety of proteins that participate in signaling transduction pathways and in other fundamental cellular functions that determine cell life or death. This review article addresses first the central aspects of calcium and redox signaling pathways in animal cells, and continues with the molecular mechanisms that underlie crosstalk between calcium and redox signals under a number of physiological or pathological conditions. To conclude, the review focuses on conditions that, by promoting cellular oxidative stress, lead to the generation of abnormal calcium signals, and how this calcium imbalance may cause a variety of human diseases including, in particular, degenerative diseases of the central nervous system and cardiac pathologies. *Antioxid. Redox Signal*. 10, 1275–1312.

I. INTRODUCTION

Localized or global increments in intracellular calcium concentration ($[Ca^{2+}]i$) have the potential to modulate redox signaling pathways in eukaryotic cells. Similarly, variations in local or global concentrations of reactive oxygen species (ROS) or nitrogen species (RNS) can affect cellular calcium-signaling pathways through different mechanisms, as described below. The relevance of this crosstalk between calcium and redox signaling for normal or pathological cell function is becoming increasingly apparent, as discussed in recent reviews that address this new aspect of cell signaling (142, 162, 434). The interactions between calcium and ROS/RNS signaling systems can be either stimulatory or inhibitory, depending on cell type, the molecular nature of the targets, the type of ROS/RNS involved, plus the duration of both signals and their concentrations and localization, as analyzed further in the text. Thus, as discussed in Section III, many studies have shown that calcium is essential for ROS/RNS production since elevation of intracellular calcium level activates enzymes that either generate or remove ROS/RNS, and enhances the formation of free radicals by the mitochondria respiratory chain. Conversely, ROS/RNS modulate proteins involved in calcium homeostasis and signaling and alter, consequently, the characteristics of both local and global calcium signals. As more information becomes available, the complex aspects of calcium and ROS/RNS crosstalk are becoming increasingly apparent. Moreover, abnormal functioning of either system may influence the other with negative repercussions for normal cell function, as exemplified by the negative effects of oxidative stress on calcium signaling analyzed in Section V.

The first section of this review presents some of the essential features of calcium signaling and homeostasis in animal cells. A succinct description of the physiological sources of ROS and RNS follows, with a focus on calcium-induced changes and crosstalk between ROS/RNS-generation. The next section details how ROS and RNS affect several cellular processes by inducing redox modifications of some of the proteins involved in calcium homeostasis and signaling. The review concludes with a description of how oxidative/nitrosative stress may cause abnormal calcium homeostasis and signaling, which may induce in turn pathological cellular states and cell death. The wide scope of topics covered in this review makes it unfeasible to address all issues with the same depth and prevents mentioning all the original reports. Accordingly, we chose to emphasize the effects of ROS/RNS on calcium signaling and homeostasis; to limit the number of references, we cited recent reviews for each subject matter whenever possible.

II. CALCIUM HOMEOSTASIS AND SIGNALING

Calcium is a universal second messenger. Calcium signals represent increases in $[Ca^{2+}]i$ over its basal intracellular level that, by modifying an array of diverse pathways, affect a variety of cellular functions (43). Calcium signals initiate very fast processes that occur in the microsecond to the millisecond time range—such as secretion of neurotransmitters and muscle contraction—and also elicit cellular events that take place in hours, such as gene transcription and cellular proliferation (29). An

excessive elevation of $[Ca^{2+}]i$ is damaging to cells and may even result in cell death. To avoid the potential harmful effects of uncontrolled intracellular calcium signals, cells make use of different regulatory mechanisms to restrain their magnitude and duration. Thus, in addition to the activation of different transporters to remove calcium from the cytoplasm, inactivation of calcium entry/release channels also limits the duration and magnitude of cellular calcium signals. Cells also employ a combination of intracellular calcium buffers and homeostatic mechanisms that comprise pumps and exchangers to return $[Ca^{2+}]i$ to its basal level following stimulation (29, 58). It is important to keep in mind—especially in the case of calcium signals generated via plasma membrane calcium channels—that ion fluxes through calcium channels are several orders of magnitude larger than fluxes through calcium transporters. Accordingly, transporters can return $[Ca^{2+}]i$ to its resting level only after calcium channel inactivation or closure; this aspect acquires special relevance in the case of brief calcium signals, such as those responsible for neurotransmitter release or striated muscle contraction.

Intracellular calcium signals can arise from calcium entry through plasma membrane pathways or via calcium release from intracellular stores; depending on their origin, calcium signals can vary in amplitude, frequency, or both. The presence of high affinity intracellular calcium binding proteins severely limits calcium diffusion throughout the cytoplasm. As a consequence, when calcium signals remain restricted to a particular cellular location, they originate what are known as calcium microdomains (26). Yet, by means of Ca^{2+}-induced Ca^{2+}-release (CICR) calcium signals can propagate in regenerative fashion to the entire cell, giving raise to global calcium signals. Therefore, the CICR process plays a very important role in calcium signaling: it not only amplifies calcium entry signals but also allows their regenerative propagation throughout the cytoplasm. The functional consequences of these different features of calcium signals depend on cell type and calcium source, among other variables (316). In particular, highly localized calcium microdomains are especially important for pre- and postsynaptic events in neurons and for heart muscle contraction. Calcium activation of calcium release channels present in intracellular stores is a key positive feedback element in the regenerative propagation of calcium signals via CICR. Moderate elevations of $[Ca^{2+}]i$ facilitate the opening of the two main types of calcium release channels present in the endoplasmic reticulum: the inositol 1,4,5-trisphosphate receptors (IP_3R) and the ryanodine receptors (RyR) (35, 114, 120, 184). In different cell types, CICR accounts for the propagation of calcium waves generated by stimulation of IP_3R (233) or RyR (380). As discussed below, ROS/RNS can modify CICR; these redox modifications can have significant effects for cellular functions that require amplification or propagation of calcium entry signals.

Resting cells have cytoplasmic $[Ca^{2+}]$ in the range of $[0.5–1.0] \times 10^{-7}$ *M*, four orders of magnitude lower than the extracellular free $[Ca^{2+}]$, which ranges from 1.0 to 1.4×10^{-3} *M*. The resulting chemical gradient across the cell membrane, combined with the negative resting membrane potential, make up a very large electrochemical gradient that provides a significant driving force for spontaneous calcium entry into cells whenever a calcium entry pathway is available. Moreover, albeit in resting cells the plasma membrane permeability to calcium is quite low, this large electrochemical gradient favors a basal level of calcium entry through leak pathways of so far unknown molecular nature, which probably comprise different molecular entities that remain undefined. The balance between calcium entry and calcium removal from the cytoplasm, including the periods when a calcium signal emerges, determines the immediate intracellular $[Ca^{2+}]$ (29). Yet, as pointed out elsewhere (336), the long-term steady-state cytoplasmic $[Ca^{2+}]$ depends exclusively on the equilibrium between the rates of inward calcium leak across the plasma membrane and the rates of calcium efflux. A similar balance between the rates of calcium uptake and calcium leak regulates steady state $[Ca^{2+}]$ within intracellular calcium stores.

The mechanisms cells employ to maintain calcium homeostasis differ from those they use to generate calcium signals. In animal cells, it is widely accepted that the homeostatic regulation of cytoplasmic $[Ca^{2+}]$ depends primarily on the activity of the four different transporters depicted in Fig. 1: the plasma membrane Ca^{2+}-ATPase (PMCA), the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the plasma membrane Na^+/Ca^{2+} exchanger (NCX), and the mitochondrial uniporter. Yet, the contribution of other organelles such as the nucleus and the Golgi system to calcium homeostasis and calcium signaling is emerging, as detailed below. Both the PMCA and the SERCA carry out primary active transport coupled directly to ATP hydrolysis; there is significant information, including data on the enzyme crystalline structure, on the mechanism used by the SERCA to move calcium ions against their chemical gradient. Essentially, calcium-dependent phosphorylation of an aspartic acid residue induces SERCA conformational changes through which the enzyme first occludes calcium ions from the cytoplasm (two calcium ions are occluded per molecule of ATP hydrolyzed), and then delivers them into the sarcoplasmic reticulum (SR) lumen (58). In addition to the SERCA and the PMCA, the NCX and the mitochondrial uniporter can also transport calcium when its cytoplasmic concentration reaches the μM range. The classical NCX is an electrogenic transporter present in the plasma membrane of excitable cells that uses the Na^+ gradient to move three Na^+ ions inside cells in exchange for one Ca^{2+} ion moving out, whereas the mitochondrial membrane potential provides the driving force for the mitochondrial calcium uniporter. These four transporters possess different thresholds for calcium activation and display different maximal transport rates. The PMCA displays the lowest rate of transport but the highest affinity, and thus it has an important role in maintaining the basal resting $[Ca^{2+}]i$ (146). The SERCA has higher transport rates and somewhat lower affinity for calcium than the PMCA, but it can still respond to rather modest elevations of cytoplasmic $[Ca^{2+}]$ (311). In contrast, the NCX and the mitochondrial uniporter have much higher transport rates but significantly lower affinities for calcium; these characteristics probably limit their contribution as calcium removal agents to conditions where cells generate large calcium signals in their immediate vicinity (146, 348).

As stated above, cytoplasmic calcium signals originate either from calcium entry or from calcium release from intracellular stores, as illustrated in Fig. 2. Depending on cell type (excitable *versus* nonexcitable cells), animal cells express in their plasma membrane a variety of well-characterized calcium channels that are gated by voltage, agonists, or second messengers (64, 273).

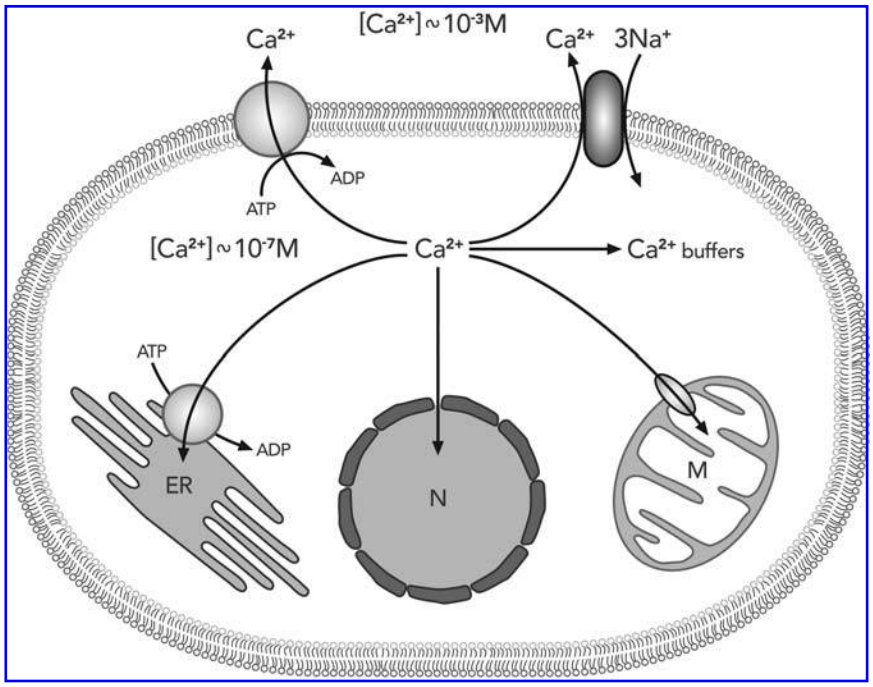

FIG. 1. Cellular components involved in calcium homeostasis. The plasma membrane contains a calcium pump (PMCA) with low transport rates but high affinity for calcium, and a sodium/calcium exchanger (NCX) with significantly lower affinity but much higher transport rates, that utilizes the energy derived from sodium entry into cells to transport calcium out of cells. Depending on the respective electrochemical gradients for sodium and calcium ions, the exchanger can also operate in the opposite direction. In addition, cells possess intracellular compartments, the endoplasmic reticulum (ER), the nucleus (N), and the mitochondria (M) that contribute to remove calcium from the cytoplasm. The ER calcium pump (SERCA) transports calcium at higher rates than the PMCA, but has somewhat lower affinity for calcium than the PMCA. Less information is available regarding the proteins responsible for calcium transport into the nucleus and the mitochondria, as discussed in the text.

Cell stimulation promotes calcium entry mainly through these plasma membrane calcium channels, although under certain conditions calcium entry via the NCX also takes place in cardiac muscle (38) or brain cells (339). Other stimuli—including G proteins, temperature, and mechanical stretch—activate a heterogeneous group of calcium channels which include the transient receptor potential (TRP) channels (285). Most cells also possess a mechanism of calcium entry activated by calcium store emptying known as store-operated calcium entry (SOCE) or capacitative calcium entry. Although initial reports described the properties of SOCE channels present in the plasma membrane of nonexcitable cells, mounting evidence indicates that SOCE channels also operate in excitable cells (306). Recent reports have shed new light on the molecular mechanisms responsible for SOCE channel activation following store emptying, and two new proteins that reconstitute SOCE function (Orai1 and STIM) have been identified (365, 385, 443).

As mentioned above, two classes of intracellular calcium channels—IP_3R and RyR—mediate calcium release from the endoplasmic reticulum (ER) and the SR. Mammalian cells possess three different isoforms for IP_3R and RyR, all coded by different genes. Receptor activation of phosphoinositide-specific phospholipase C (PLC) generates inositol 1,4,5-trisphosphate (IP_3), which binds to IP_3R, activates channel opening and generates calcium signals in almost every cell type (27, 72, 191, 280). The RyR calcium release channels were named as such because they bind selectively and with high affinity (in the 10^{-9} *M* range) the plant alkaloid ryanodine, a property that allowed their initial identification and purification from SR vesicles isolated from skeletal muscle (228). RyR channels are also present in many other cell types, including excitable and nonexcitable cells (119). Depending on cell type and RyR isoform, different signals, including calcium itself, plasma membrane depolarization, cyclic ADP-ribose (cADPR), and ROS/RNS activate RyR. The signals that switch off calcium release channels vary, depending on channel type and isoform; yet, in many cases a cytoplasmic increase or a luminal decrease in $[Ca^{2+}]$ turns these channels off.

III. ROS/RNS SIGNALING

Overall, ROS and RNS, including nitric oxide (NO) gas, are short-lived small molecules, that, depending on their reactivity, can reach only immediate targets or can diffuse freely within cells or even outside cells. Thus, although short-lived, NO can diffuse within tissues and cell cultures to longer distances than hydroxyl radicals, peroxynitrite, and peroxynitrite-derived radicals, that have much shorter lifetimes within cells, and than superoxide anion, which shows a very poor diffusion coefficient across the lipid bilayer compared with NO. It has become increasingly apparent that ROS/RNS serve a function as signaling molecules in various biological responses, that include gene expression and cell death, among others (355). Thus, the early perception that ROS/RNS are toxic to cells has been replaced by a new paradigm whereby at low concentrations ROS/RNS serve a physiological role as second messengers, while at high concentrations they induce oxidative or nitrosative stress. A historical perspective on the changing role of ROS/RNS, from toxic agents to signaling molecules, is presented in detail elsewhere (98, 99, 230). Briefly, evidence supporting a second messenger role for ROS began to emerge in the late 1970s, with two reports in mammalian cells describing activation of guanylate cyclase by ROS derived from superoxide anion (283, 419). These early findings were followed a decade later by the inde-

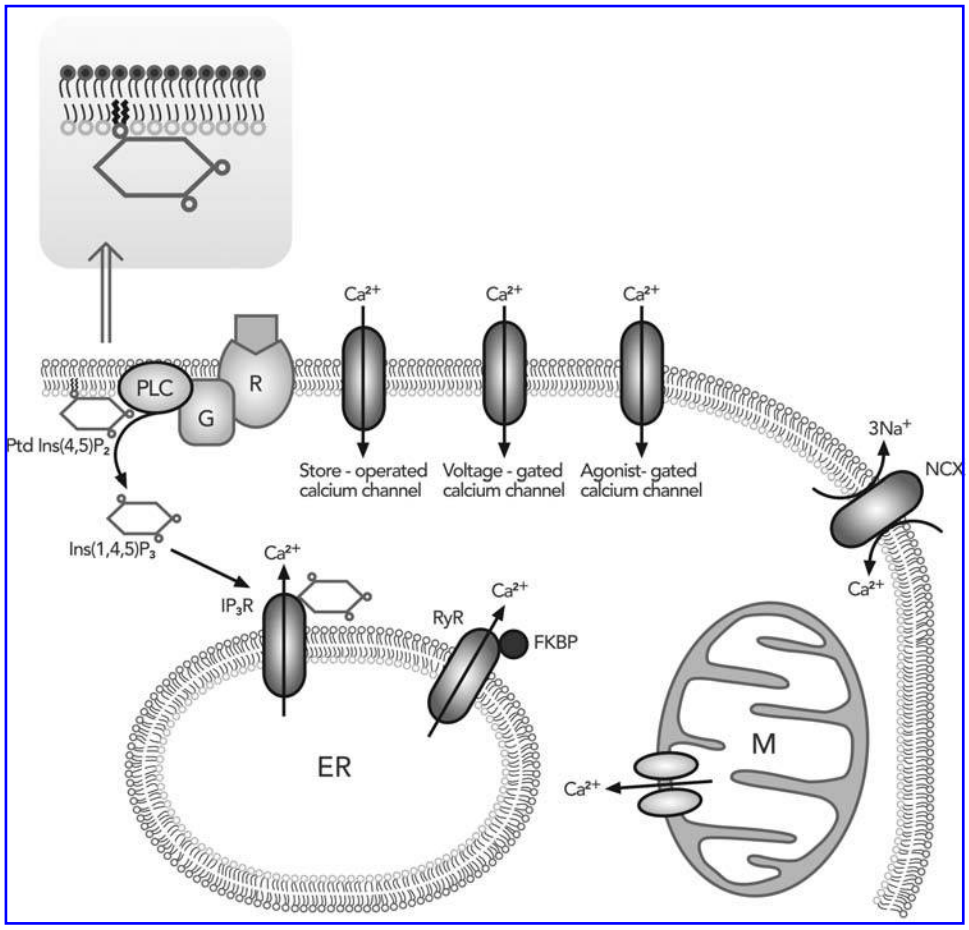

FIG. 2. Cellular pathways that contribute to increase $[Ca^{2+}]i$: Excitable and nonexcitable cells generate calcium signals in response to a variety of stimuli that depend on cell type. These calcium signals arise from calcium entry into cells through different types of plasma membrane calcium channels, which include voltage- and agonist-gated calcium channels and the store operated calcium entry (SOCE) channels, or via the sodium/calcium exchanger present in excitable cells when working in the reverse mode. Calcium signals also originate via calcium release from the endoplasmic reticulum, through IP_3R or RyR calcium release channels that are present in most cell types. Agonist binding to plasma receptors stimulates via a trimeric G protein the hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate by PLC, generating IP_3 that diffuses through the cytoplasm until it binds to IP_3R and promotes calcium release. The activation of RyR calcium release channels is regulated by two isoforms of proteins that bind the immunophilin FK506 (FKBP), and depends on cell type and RyR isoform as discussed in detail in the text. Mitochondria also contribute to the generation of calcium signals. Calcium ions move out of the mitochondria through the mitochondrial Na^+/Ca^{2+} exchanger or a Na^+-independent system; the calcium uniporter and the mitochondrial permeability transition pore may represent alternative routes for calcium exit.

pendent discovery of the key role of NO in smooth muscle relaxation (183) and platelet adhesion (330). At this time, it was also reported that hydrogen peroxide increases T-cell production of interleukin-2, a growth factor important for the immune response (341), induces the expression of the heme oxygenase gene (210), and activates the transcription factor NF-κB (352). In those years, reports that ROS/RNS regulate the function of ion channels involved in calcium signaling began to emerge. To cite a few examples, it was shown that exogenous or endogenous NO directly activates single Ca^{2+}-dependent K^+ channels in cell-free membrane patches from vascular smooth muscle cells (39), whereas ROS-induced activation of IP3R and RyR calcium release channels, which has distinct implications for crosstalk between ROS and calcium signaling, was first reported in 1989–1990 (313, 327, 437, 438). Further evidence supporting a role for ROS as second messengers came from studies showing that addition of platelet-derived growth factor (PDGF) to vascular smooth muscle cells increased intracellular hydrogen peroxide levels, whereas increasing the intracellular levels of catalase or glutathione prevented the ROS increase and blocked PDGF-induced signaling (372).

Intracellular glutathione (GSH), which in animal cells lies in the m*M* range, has a central role in cellular redox signaling (278). By forming part of the glutathione disulfide/glutathione (GSSG/GSH) redox pair, GSH makes an important contribution towards the maintenance of cellular redox state, since this pair maintains the redox state of protein thiols and contributes to reduce oxidized thiols. Together with other enzymatic and nonenzymatic systems, GSH is central for cellular redox homeostasis and antioxidant defense mechanisms (Fig. 3). Addi-

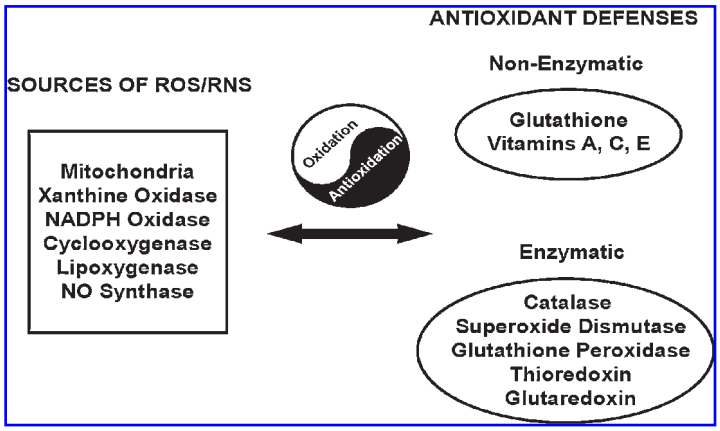

FIG. 3. Sources of ROS/RNS and cellular antioxidant defense systems. The mitochondria and several cellular enzymes generate ROS/RNS. Cells maintain constantly their redox balance through the action of nonenzymatic and enzymatic defense systems, such as those illustrated in the figure.

tionally, through *S*-glutathionylation of cysteine sulfhydryl (SH) groups (formation of protein–S-SG intermediates), GSH modulates the activity of a variety of proteins. Two different enzyme types catalyze the exchange between GSH and GSSG: GSH peroxidases catalyze the oxidation of GSH to GSSG, whereas the NADPH-dependent GSH reductase catalyzes the reduction of GSSG to GSH (97). GSH also provides cellular protection against oxidative damage by reacting with RNS, including NO and peroxynitrite, to form *S*-nitrosoglutathione (GSNO). Yet, as discussed below in Section V, high levels of oxidative/nitrosative stress can lead to GSH depletion with the consequent threat for cell survival.

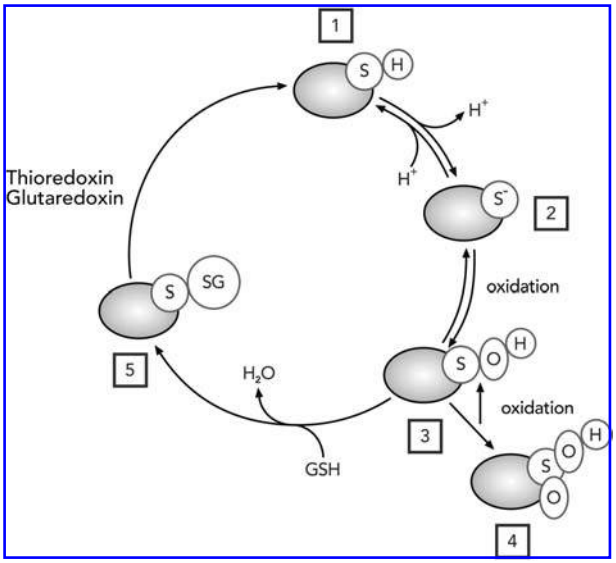

FIG. 4. Covalent modifications of cysteine residues by ROS. The free thiol group of cysteine residues [*step 1*] undergoes reversible dissociation, releasing protons [*step 2*]. The dissociated form can react with oxidants to generate the sulfenic derivatives, which are reversible oxidation products [*step 3*]. The sulfenic derivatives can be further oxidized irreversibly to sulfinic [*step 4*] or sulfonic derivatives, or can undergo *S*-glutathionylation [*step 5*] by reacting with GSH. The cycle is completed by thioredoxin or glutaredoxin, which return the modified SH residue to the free thiol form.

Post-translational modifications by ROS/RNS modify the function of many cellular proteins involved in signal transduction, such as protein phosphatases, protein/lipid kinases, and transcription factors, with significant consequences for overall cellular function. Cysteine residues are the typical targets of redox modifications, albeit other amino acids can also react with ROS/RNS. This review, however, places its main emphasis on the functional consequences of redox modifications of protein cysteine residues. As illustrated in Fig. 4, the free thiol group of cysteine can undergo reversible or irreversible covalent modifications by ROS, that can induce the sequential generation of reversible or irreversible oxidation products, such as sulfenic, sulfinic, and sulfonic derivatives. Additionally, ROS/RNS can promote the reversible *S*-nitrosylation or *S*-glutathionylation of cysteine SH residues and induce disulfide formation. This last modification prevents further reaction of SH groups with ROS/RNS (Fig. 5).

Both superoxide anion and hydrogen peroxide appear to target with relative specificity reduced SH groups of cysteine residues within proteins. In addition to its well-known stimulation of guanylyl cyclase, NO or NO-derived RNS also induce protein modifications through *S*-nitrosylation of cysteine residues. The resulting post-translational protein modifications can exert positive or negative regulation over a variety of cellular signaling pathways and metabolic processes. As an example, *S*-nitrosylation inhibits the activities of the transcription factor NF-κB (267), protein kinase C (194), and JNK (307), but activates Ras (231) and RyR, as discussed in detail below. Whether NO, which seems to be an inefficient *S*-nitrosylating agent, or other low molecular weight nitrosothiols, cause these effects is currently a subject of active investigation (44).

Reversible *S*-glutathionylation is a relatively widespread form of modulation of the activities of proteins containing redox-sensitive thiols, including those involved in signal transduction pathways. Cellular GSH abundance, combined with the ready conversion of *S*-nitroso and sulfenic acid derivatives into *S*-glutathione mixed disulfides, strongly suggest that reversible protein *S*-glutathionylation is a central mechanism of redox signal transduction (358).

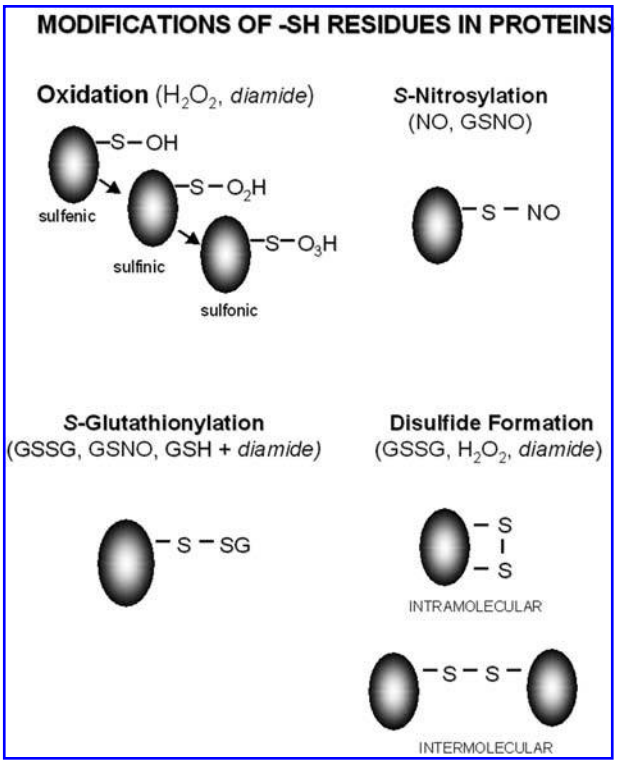

FIG. 5. Modifications of sulfhydryl residues in proteins by ROS/RNS. Endogenous or exogenous ROS/RNS can induce a range of different modifications of protein thiol groups, which include oxidation to sulfenic, sulfinic, and sulfonic derivatives, *S*-nitrosylation, *S*-glutathionylation, and the formation of intra or intermolecular disulfide bonds.

Cells possess enzymatic systems that act as antioxidant defenses (Fig. 3) as well as enzymes that regulate the functional changes induced by ROS/RNS, including glutaredoxin, thioredoxin, glutathione *S*-transferase, peroxiredoxin, superoxide dismutases (SOD), and sulfiredoxin. There are three mammalian SOD isoforms: mitochondrial SOD (SOD-2 or Mn-SOD), cytoplasmic SOD (SOD-1 or Cu/Zn-SOD), and extracellular SOD (EC SOD) (190). Glutaredoxin acts as a specific and efficient catalyst of protein de-glutathionylation reactions (358). Animal cells contain multiple glutaredoxins, which catalyze disulfide reduction in the presence of NADPH, GSH, and glutathione reductase (the glutaredoxin system). In addition to the glutaredoxins, thioredoxin can also reduce reversibly modified thiols. The thioredoxin system maintains the activity of peroxiredoxins and methionine sulfoxide reductases, which have important antioxidant roles. Glutaredoxins, like thioredoxins, are also involved in cellular functions, including the defense against oxidative stress (117). Some isoenzymes of the super family of glutathione *S*-transferases can also regulate mitogen-activated protein kinases or facilitate protein *S*-glutathionylation (271). Other enzymes, such as SOD, peroxiredoxin, and sulfiredoxin, contribute to maintain redox homeostasis. Sulfiredoxin is a recently identified antioxidant protein that promotes the reduction of a conserved peroxiredoxin(s) cysteine residue from sulfinic to sulfenic acid, preventing its further oxidation to sulfonic acid that leads to peroxiredoxin degradation. In addition, sulfiredoxin catalyzes nonspecific de-glutathionylation of proteins (121).

As pointed out recently (332), it is important to consider how ROS/RNS can act as second messengers in the presence of all these antioxidant cellular enzyme systems. Localized generation of ROS/RNS in specific subcellular compartments may protect redox-induced modifications at least transiently from the action of antioxidant enzymes, as happens for NOX-generated ROS (402). Alternatively, inactivation of antioxidant enzymes may represent another cellular strategy for effective ROS/RNS signaling. An example of this mechanism is the H_2O_2-induced inactivation of peroxiredoxin during the catalysis of H_2O_2 reduction, which can be reversed by sulfiredoxin (332).

A. Physiological sources of ROS and RNS

Mammalian cells contain several sources of ROS; these include the mitochondria and cytoplasmic or membrane-bound enzymes such as the NADPH oxidases (NOX), xanthine oxidase (XO), cyclooxygenase (COX), and lipoxygenase (LOX). Cells also generate NO via nitric oxide synthases (NOS); nonenzymatic reaction of NO with superoxide anion or GSH generates peroxynitrite or GSNO, respectively. The next sections analyze the effects of these different ROS/RNS on calcium signaling under physiological or pathological conditions.

The respiratory conditions of mitochondria determine the amount of superoxide generated and its site of generation (55). In normal conditions, the mitochondria electron transport chain produces superoxide anion in respiratory complexes I and III; at low electron transport rates the main source of superoxide is complex I, while in fully respiring mitochondria (state 3) the main source of superoxide anion is complex III. An increase in mitochondrial [Ca^{2+}] enhances ROS production in certain metabolic situations, whereas in other conditions (state 4) calcium-induced dissipation of mitochondrial potential could minimize ROS production (55). Mitochondrial and cytoplasmic SOD transform superoxide into hydrogen peroxide (H_2O_2), whereas catalase and glutathione peroxidase reduce H_2O_2 to water, as summarized in the scheme illustrated in Fig. 6. Superoxide anions escape to the cytoplasm—presumably via anion channels—when their concentration in the mitochondrial matrix reaches high enough levels. On the other hand, H_2O_2 apparently diffuses freely through the inner and outer mitochondrial membranes and reaches the cytoplasm, where it can modify cellular proteins (37, 332) or generate hydroxyl radical in the presence of iron or copper ions in their reduced states (55). Recent evidence indicates that H_2O_2 may also cross membranes that are poorly permeable to it through aquaporins, the channels that facilitate the diffusion of noncharged solutes such as water (37).

The NADPH oxidase enzymes, first identified in phagocytic cells, catalyze the transfer of one electron from NADPH to oxygen to produce superoxide anion. The phagocytic NOX possesses two integral membrane subunits that form its catalytic core, $gp91^{phox}$ (NOX2) and $p22^{phox}$, and four cytoplasmic regulatory subunits, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase Rac. Seven genes that differ in mRNA tissue expression encode the NOX isoforms NOX1-5 and Duox1-2 (65, 229, 402). Current knowledge indicates that virtually every cell and

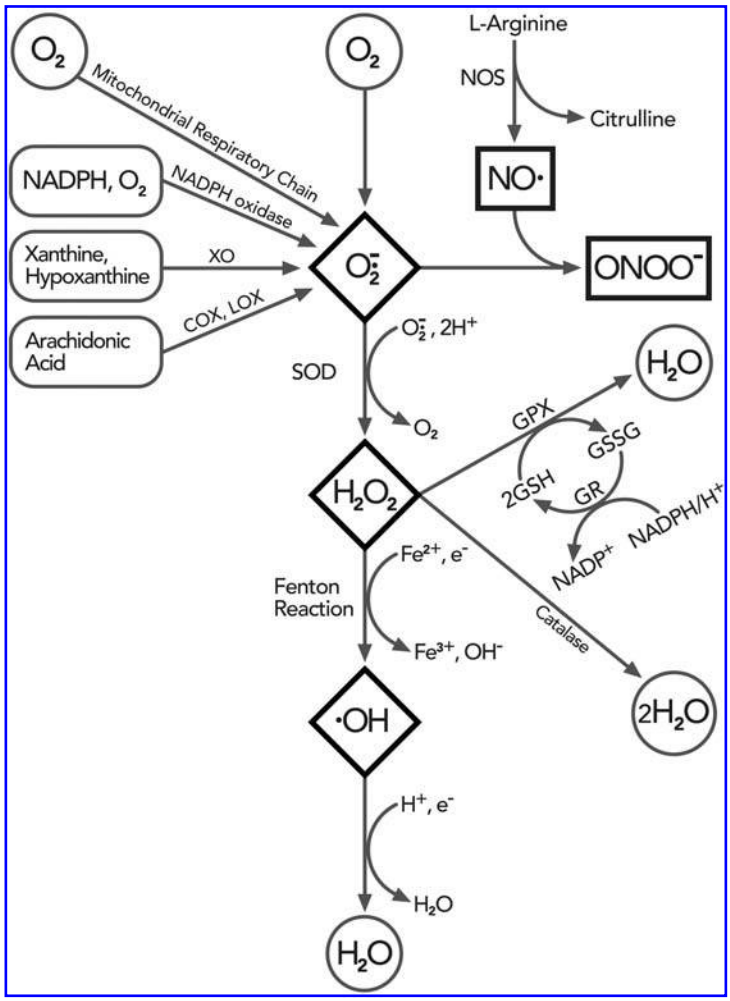

FIG. 6. Cellular pathways of ROS/RNS formation. The mitochondrial respiratory chain and enzymes systems that include the NADPH-oxidase (NOX), xanthine oxidase (XO), cyclooxygenase (COX), and lipooxygenase (LOX), generate superoxide anion from molecular O_2. Superoxide can dismutate into H_2O_2 spontaneously or enzymatically through the action of SOD, or can react with NOS-produced NO to generate peroxynitrite. The enzymes glutathione peroxidase (GPX) and catalase can convert H_2O_2 into water. Alternatively, H_2O_2 can react with redox active metal ions, such as Fe^{2+}, to generate the hydroxyl radical.

tissue expresses some type of NOX isoform, which differ in subcellular localization and regulation. The assembly of NOX proteins in lipid rafts (410) has been reported, and new evidence indicates that NOX enzymes have a role in the modulation of physiological and pathological processes (see Section V). Recent excellent reviews discuss the role of NADPH oxidases in normal and pathological conditions in the cardiovascular system (65) and other tissues (22).

Xanthine oxidase catalyzes the conversion of hypoxanthine into xanthine in the purine catabolic pathway, and of xanthine into uric acid, generating both superoxide anion and H_2O_2. In basal conditions, XO is present as xanthine dehydrogenase, an enzyme that utilizes NAD^+ instead of oxygen as electron acceptor and does not produce ROS. Under stress, many tissues transform xanthine dehydrogenase into XO by reversible oxidation of its SH groups or by irreversible proteolysis (31). XO is an important source of ROS in the liver and intestine, tissues with especially high XO activity. Cyclooxygenase and lipoxygenase are the rate limiting enzymes in the biosynthesis of prostanoids (prostaglandins and thromboxanes) and leukotrienes, respectively (100). These enzymes are presumably important sources of ROS in brain blood vessels. As described in Section V, some pathological conditions enhance XO expression and activity in cardiac muscle, whereas COX and LOX may also become important ROS sources in some pathological situations. In particular, calcium activates 5-LOX, an enzyme that catalyzes the first two steps of leukotriene biosynthesis, and significantly stimulates leukotriene generation (329).

The three mammalian NOS enzymes are cellular sources of NO, which in turn generates NO-derived RNS, including some free radicals. These enzymes use arginine and oxygen as substrates to produce NO, which can combine with superoxide anion to generate the strongly oxidant peroxynitrite anion. Various cell types constitutively express the two isoforms that are regulated by calcium and calmodulin (CaM): the neuronal (nNOS) NOS and the endothelial (eNOS) NOS. In contrast, other stimuli activate the inducible NOS isoform (iNOS) that is practically independent of calcium and CaM. The active NOS enzyme is a homodimer and all three isoforms require several cofactors for catalytic activity: NADPH, tetrahydrobiopterine (BH_4), and the flavin nucleotides FAD and FMN. In the absence of L-arginine or BH_4, NOS may become uncoupled; in this state electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other are accepted by oxygen rather than by L-arginine, resulting in the production of superoxide anion instead of NO (289).

B. Effects of ROS/RNS and calcium on ROS/RNS signaling

It is becoming increasingly apparent that there is positive feedback between the cellular enzymes that generate ROS/RNS. In hypertensive animals, NOX activation in vascular cells leads to BH_4 oxidation, which by inducing endothelial NOS uncoupling results in the production of large amounts of superoxide anion (232). Another example of positive feedback is the functional coupling between iNOS and COX-2 described in a macrophage cell line, whereby iNOS *S*-nitrosylates a particular COX-2 cysteine residue and increases its activity (212). Additionally, mitochondrial ROS-induced ROS release (450), a mechanism in which mitochondrial ROS released into the cytoplasm trigger ROS release from neighboring mitochondria (451), also constitutes a positive feedback mechanism for enhanced ROS production that may cause mitochondrial damage and cellular injury.

Moreover, calcium signals affect some of the pathways responsible for cellular ROS/RNS generation. Thus, in addition to its key role in the stimulation of calcium-dependent NOS isoforms (391), calcium stimulates the three NOX isoforms that possess N-terminal EF-hand motifs: NOX5, Duox1, and Duox2. Via two proteins of the S100 family, known as MRP8 and MRP14, calcium activates the phagocytic NOX2 isoform that lacks EF-hand motifs. These two small proteins, which contain EF-hand motifs, associate with NOX2 in response to calcium and enhance its activation synergistically with $p47^{phox}$ and $p67^{phox}$ (32). In cardiac muscle, in particular, ROS/RNS affect calcium signaling in physiological and pathophysiological situations. Increases in stimulation frequency, which augment the time average $[Ca^{2+}]i$, enhance the generation of mitochondrial ROS in isolated cardiac myocytes (159). Similarly, increasing heart rate by electrical stimulation (345) or exercise (344) increases the activity of NADPH oxidase in dog cardiac muscle, which in turn stimulates RyR2-mediated CICR in isolated SR vesicles.

IV. REDOX MODIFICATION OF PROTEINS INVOLVED IN CALCIUM HOMEOSTASIS AND SIGNALING

As already mentioned, the concept that ROS/RNS act as intracellular messengers is receiving increasing attention and has become more generally accepted (98). Cellular ROS/RNS play important roles during cell growth and differentiation (349) and modify the activity of several transport systems (222). Yet their involvement in the generation and modulation of physiological calcium signals is only recently emerging (142, 340, 388, 435). The cellular locations of calcium and ROS signal generation, both of which are under tight regulation, are decisive for their reciprocal interactions. We will address this crucial point in further detail in the following section.

The modification of cysteine residues plays an important role in redox modulation of a variety of proteins involved in calcium homeostasis and signaling, all of which display different sensitivities to redox agents. In broad terms, ROS/RNS favor the emergence of calcium signals because they impair the function of calcium pumps and stimulate calcium release from internal stores. Thus, oxidants inhibit calcium transport by the PMCA and the SERCA (but see below), while their effects on the plasma membrane NCX are controversial. Oxidants also stimulate the two main types of calcium release channels of intracellular stores, IP_3R and RyR. A more detailed analysis of the effect of redox modifications on the function of the main calcium handling cellular proteins follows.

A. Plasma membrane calcium pump and Na^+/Ca^{2+} exchanger

As described in Section II, the plasma membrane calcium ATPase or PMCA is a low capacity, high affinity calcium pump that, by controlling resting cytoplasmic $[Ca^{2+}]$, plays a central role in calcium homeostasis (58, 146). There are four different PMCA isoforms coded by four separate genes; alternative RNA splicing of the primary gene transcripts originates many variants that differ primarily in their regulatory regions. The PMCA 1 and 4 isoforms are present in almost all tissues, while isoforms 2 and 3 are restricted to more specialized cell types. An autoinhibitory domain, which blocks ATP binding and hydrolysis, regulates PMCA activity. CaM binding to a PMCA region composed by 20 amino acid residues located near its C-terminus (59) induces the dissociation of the autoinhibitory domain from the active site of the enzyme, and increases several-fold the calcium pumping rates.

Both the PMCA and CaM are sensitive to oxidative modifications. ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, inactivate the PMCA (197, 436, 437). Loss of two cysteine residues without concomitant production of sulfenic or sulfinic acids, implying disulfide bond formation, accompanies the inactivation by H_2O_2 of the PMCA purified from human erythrocyte membranes; dithiothreitol (DTT) addition, however, does not induce a recovery of PMCA activity, indicating irreversible enzyme inactivation (436). In erythrocyte membranes, CaM decreases the PMCA sensitivity to H_2O_2, suggesting that CaM binding buries the amino acid residues susceptible to oxidation (436). Both hydrogen peroxide and superoxide anion reportedly produce reversible inhibition of the PMCA activity of cardiac myocytes, since DTT and cysteine effectively prevent this inhibition (197). These findings suggest that reversible cysteine modifications underlie the ROS-induced inhibition of PMCA activity in these cells. Reactive nitrogen species such as peroxynitrite also inhibit the PMCA. In rat brain synaptosomes, the pump is irreversibly inhibited by repetitive pulses of micromolar concentrations of peroxynitrite, an effect presumably produced by irreversible nitration of enzyme tyrosine residues, albeit oxidation of cysteine residues was not excluded (148). The specific amino acids responsible for ROS-induced inactivation of the PMCA remain unidentified. Theoretical analysis using tri-dimensional modeling suggests that ROS modify PMCA residues Tyr 589, Met 622, and Met 831 (256). Recent evidence suggests that PMCA inhibition by oxidation, which may be due to faulty interactions between the ATP binding and the autoinhibitory domains, may represent an adaptive response to minimize ATP consumption during oxidative stress conditions (304). Oxidation of CaM methionine residues 144 and 145 may also contribute to the inhibition of PCMA activity induced by ROS, since fol-

lowing oxidation, CaM stabilizes the PMCA in an inactive conformation (5, 67).

In nearly all cells, the PMCA is the main system in charge of removing to the external medium the calcium ions that enter resting cells through leakage pathways. Yet the NCX present in excitable cells has an important role in lowering $[Ca^{2+}]i$ following stimulation, and may even contribute to generate calcium signals in some particular conditions such as cell membrane depolarization (38, 92). In particular, the PMCA plays a minor role in calcium extrusion in comparison to the NCX following cardiomyocyte contraction. There are three distinct genes and several splice variants for the classical NCX transporter, that catalyzes the electrogenic exchange of three sodium ions and one calcium ion across the plasma membrane. Depending on the electrochemical gradient, the exchanger can work in the direct mode resulting in calcium extrusion, or in the reverse mode producing calcium entry. In addition, bovine rod photoreceptors and the brain express NCKX, a closely related transporter that uses jointly the inward Na^+ gradient and the outward K^+ gradient for Ca^{2+} extrusion at a stoichiometry of $4Na^+/Ca^{2+}\text{-}K^+$; interestingly, charge-removing replacement of a single amino acid renders the mutant NCKX proteins independent of K^+ (198).

Conflicting reports exist regarding NCX redox sensitivity (182). In cardiac muscle NCX activity increases after exposing isolated sarcolemmal vesicles to DTT plus $FeSO_4$, or to glutathione plus glutathione disulfide, suggesting that a thiol–disulfide interchange activates the protein (331). Consistently, H_2O_2 and superoxide generated by XO enhance the NCX activity of voltage-clamped ventricular myocytes (138). Furthermore, hypoxia inhibits the NCX of cardiac myocytes, and its reactivation during reoxygenation depends on ROS (107). Other results (346), however, raise doubts on the role of disulfide bond cycling in redox regulation of the NCX. While treatment with DTT and $FeSO_4$ stimulate NCX activity by removing Na^+-dependent inactivation, a mutant NCX protein that lacks cysteine residues (Cys-less) still shows Na^+-dependent inactivation. Moreover, reintroduction of cysteines (C14, C20, and C792) into the Cys-less NCX induced a recovery of redox activation, suggesting that these are the cysteines involved in thiol disulfide rearrangement; however, Fe-DTT still stimulates NCX activity after elimination of any one of these residues. These results make unlikely the contribution of disulfide rearrangement to this stimulation, which takes place via mechanisms that remain unknown (346). A similar Cys-less mutant protein for the NCKX displays similar behavior as the cardiac NCX since partial or total replacement of its cysteine residues does not have a critical impact in its activity (215).

B. Plasma membrane voltage-gated and store-operated calcium channels

Voltage-gated Ca^{2+} channels are heteromultimeric complexes—with a major pore-forming $\alpha 1$ subunit and at least four other subunits (β, $\alpha 2/\delta$. and γ)—that mediate calcium influx in response to membrane depolarization and that contribute to induce diverse cellular responses such as contraction, secretion, and gene expression. There are several calcium channel types known as L-type ($Ca_v1.1$-1.4), N-type ($Ca_v2.2$), P/Q-type ($Ca_v2.1$) , R-type ($Ca_v2.3$), and T-type ($Ca_v3.1$-3.3) that differ in their physiological and pharmacological properties (63).

Exogenous and endogenous redox compounds affect the activity of L-type Ca^{2+} channels (174), but there is no general agreement on the effects of oxidation on channel activity. Among the earlier reports supporting L-type channel inhibition by oxidants is a study showing that oxidation of SH groups (presumably present in the channel protein) induces dysfunctional calcium entry in isolated guinea pig ventricular myocytes by altering the channel gating process (226). Likewise, SH modifications with exogenous reagents inhibit calcium currents through the cardiac pore-forming $\alpha 1$ subunit expressed in a heterologous system (HEK293 cells); DTT reverses this inhibition, suggesting that channel gating requires free SH groups (71). ROS generated by xanthine plus XO also inhibit Ca^{2+} currents through L-type calcium channels in isolated cardiac myocytes (135, 147, 394), but the molecular basis underlying these effects is unknown.

In contrast to the above findings, it has been reported that an exogenous thiol reducing agent decreases basal L-type calcium currents in ventricular myocytes whereas oxidizing agents increase them (172). Since these effects persist even when cAMP production or G-protein is inhibited, it was concluded that L-type calcium channels possess some sites that are subject to direct modification by SH reagents (433). A recent study (414) indicates that transient exposure of cardiac myocytes to H_2O_2 increases intracellular ROS and basal L-type channel activity, and increases for several hours diastolic calcium in quiescent and contracting myocytes. Since L-type calcium channel inhibition attenuates ROS production, it was proposed that H_2O_2 enhances mitochondrial superoxide anion generation via stimulation of calcium influx. Likewise, a separate study of the pore-forming cardiac $\alpha 1$ subunit stably expressed in HEK 293 cells also indicates that H_2O_2 stimulates basal L-type channel activity (181).

In isolated cardiac myocytes, NO shows dual effects: it activates L-type calcium channels in a DTT reversible manner and it inhibits them by a cGMP-mediated process (56). According to these authors, *S*-nitrosylation of extracellular channel SH residues would underlie this inhibition. Yet, this proposed covalent modification of the channel protein has not been confirmed experimentally. More recent work indicates that GSNO, presumably via *S*-nitrosylation of intracellular SH groups, inhibits the gating and conductance of the $\alpha 1$ subunit of smooth muscle L-type calcium channels (321).

In addition to redox agents and NO, hypoxia also modifies L-type calcium channel activity. It has been reported that hypoxia causes reversible inhibition of the currents mediated by L-type Ca^{2+} channels in vascular cells (125). This inhibition can be prevented by *p*-chloromercuribenzoate (115), suggesting involvement of channel cysteine residues. The oxygen sensitivity of the $\alpha 1$ channel subunit resides in a 37-amino acid region (116). but the molecular mechanisms detailing why this region confers oxygen sensitivity to the channel protein remain to be established. Hypoxia also decreases H_2O_2 production and basal L-type mediated calcium currents, and enhances the responsiveness of cardiac L-type Ca^{2+} channels to stimulation by β-adrenergic agonists (173). Yet, neither altered H_2O_2 production nor regulation of the ROS-producing NADPH oxidase un-

derlie the inhibition of L-type Ca^{2+} channels produced by hypoxia (181).

In contrast to the inhibition observed in vascular and cardiac muscle cells, hypoxia increases calcium currents through L-type calcium channels in PC12 cells and cerebellar granule neurons; HEK 293 cells display this same behavior after heterologous expression of the human cardiac L-type calcium channel $\alpha 1$ subunit (310). These effects of hypoxia have been ascribed, however, to the upregulation of calcium channels induced by increased formation of the β amyloid peptide, and not to direct redox modifications of the channel $\alpha 1$ subunit (310).

Limited information is available on the effects of redox agents for the other voltage-gated Ca^{2+} channel types. Oxidation by external H_2O_2 accelerates the overall opening process of single neuronal P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes (238). In contrast, reducing agents such as L-cysteine and DTT enhance T-type currents in rat sensory neurons, whereas exogenous oxidizing agents inhibit them. These effects of redox active compounds on T-type currents correlate with the decrease in thermal and mechanical pain thresholds induced by reducing agents and the opposite effects of oxidizing agents. Pain perception modulation by endogenous or exogenous redox agents emerges as an interesting possibility (36, 295, 308, 393).

To summarize, several reports indicate that oxidation increases calcium influx through voltage-dependent calcium channels. Yet, other reports indicate that oxidizing agents inhibit voltage-gated Ca^{2+} channel activity, and thus may prevent depolarization-induced calcium entry into excitable cells under conditions of excessive ROS production (242). This hypothesis, however, requires additional experimental information to ascertain its general validity. Furthermore, the effects of hypoxia, which has been associated with increased ROS production, depend on the mechanisms displayed by different cell types to cope with this condition.

Current information on redox modification of store-operated calcium entry and TRP channels is also rather limited. Hydrogen peroxide inhibits SOCE in thyroid cells, but this inhibition seems to be caused through activation of PKC and not by redox modulation of SOCE channels (334, 395). In addition, there is evidence that NO decreases SOCE in isolated small pulmonary arteries via PKG-dependent inhibitory effects, which are absent after chronic hypoxia (188).

Hydrogen peroxide and other agents that produce ROS/RNS activate the widely expressed melastatin-related transient receptor potential TRPM2 channel (157), a plasma membrane cation channel permeable to Na^+ and Ca^{2+} that causes membrane depolarization and increases $[Ca^{2+}]i$. Although some authors suggest that channel activation is indirect and takes place via the effects of H_2O_2 on mitochondria (312), other results indicate that oxidative stress activates TRPM2 channels, which contribute to insulin secretion in pancreatic β cells when stimulated by hydrogen peroxide (108). Covalent modifications of reactive cysteines within TRPA1 channels present in peripheral nociceptive neurons, which sense noxious stimuli such as cold temperatures, irritating environmental chemicals and pungent natural compounds, activate these channels; of the fourteen cytoplasmic cysteines that bind SH-directed reagents, only three are required for channel function (260). These results indicate that the nervous system can sense peripheral damage through TRPA1 activation, induced via redox modifications of specific channel cysteine residues by noxious stimuli.

C. Agonist-gated plasma membrane calcium channels

The N-methyl D-aspartate (NMDA) receptor is a channel with high permeability to calcium that in physiological conditions is jointly activated by glutamate/glycine and membrane depolarization. In the hippocampus, calcium entry through NMDA receptors (heteromeric complexes composed of at least one NR1 subunit and one or more of the two subunits, NR2A and NR2B) is an important determinant of several neuronal functions including synaptic plasticity, learning, and memory (258). Yet, excessive activation of NMDA receptors can cause excessive Ca^{2+} influx, which contributes to several degenerative neuronal disorders and may result in neuronal death (247). Therefore, adequate regulation of NMDA receptor activity is crucial for neuronal survival and activity.

NMDA receptors undergo diverse modifications by ROS and RNS that modulate their activity. The redox modifications of NMDA receptors include redox modulation of disulfide bond formation and *S*-nitrosylation of free thiol groups on critical cysteine residues (75, 234, 245, 246). Reducing agents such as DTT enhance NMDA receptor activity by promoting the formation of free thiols; in contrast, oxidizing agents, including endogenous GSSG, inhibit NMDA evoked currents by promoting disulfide bridge formation (3, 13, 74, 219, 220, 246, 351, 384). Likewise, significant modifications of the NMDA receptor take place following its phosphorylation by protein tyrosine kinases (220, 417) or by serine/threonine kinases (302, 392). We point this out because the phosphatases that act on phosphorylated tyrosine or serine/threonine residues are also redox sensitive (13, 294, 416) and thus they may represent an additional albeit indirect mechanism of redox regulation of NMDA receptor activity.

Nitric oxide is also an important endogenous modulator of NMDA receptors. NO donors such as *S*-nitrosocysteine inhibit NMDA-evoked currents in primary cortical neurons by inducing *S*-nitrosylation and disulfide bond formation of critical NMDA receptor cysteine residues (234, 245, 247); NR1/NR2A recombinant receptors expressed in heterologous cells exhibit similar behavior (75, 246). Modification of NMDA receptor SH groups with specific reagents abolishes the inhibition produced by NO (213).

Several cysteine residues, identified by site-directed mutagenesis, are responsible for NMDA receptor redox modulation. These cysteines form disulfide bonds within both the NR1 receptor subunit (C79 and C308; C744 and C798) and the NR2A subunit (C87 and C320) (74, 75). Furthermore, site-directed mutagenesis has identified C399 on the NR2A subunit of the NMDA receptor as the cysteine responsible for the inhibition of receptor activity upon nitrosylation (75). As recently shown, the sensitivity of NMDA receptors to nitrosylation resides in cysteines C744 and C798, which act as redox sensors (379). When these cysteines form part of a disulfide bridge, nitrosylation of C399 only occurs at very high NO concentrations. In contrast, in the initial states of hypoxia that favor the reduction of disulfides to free thiols, C744 and C798 are in the free thiol

state and can be *S*-nitrosylated by low NO concentrations. Following *S*-nitrosylation, they exert an allosteric influence that favors the nitrosylation of C399 that produces the inhibition of NMDA receptors. This mechanism, illustrated in Fig. 7, would prevent excessive NMDA receptor activity and citotoxicity and thus may be particularly important during hypoxia or stroke conditions (379).

D. Other ion channels related to calcium signaling

In addition to the calcium channels described above, a number of other ion channels that influence calcium signaling are sensitive to redox agents (222). Hydroxyl radical activates a calcium-sensitive nonselective cation channel that is involved in necrosis of epithelial cells (362). Additionally and depending on the oxidative species, ROS can activate, inhibit, or leave unaltered voltage-gated potassium channels in blood vessels (149).

Redox agents also modify the activity of high conductance calcium activated potassium channels (BKCa), which in rat brain form complexes with L-type, P/Q type, and N-type voltage-gated calcium channels (25). Several reports indicate that ROS/RNS exert different effects on BKCa channels, which apparently depend on cellular type. Thus, while NO stimulates vascular smooth muscle BKCa channels (39), thimerosal and other exogenous SH reagents inhibit them in bovine aortic endothelial cells (54). Moreover, superoxide anion and hydrogen peroxide enhance BKCa channel activity in rat and cat cerebral arterioles, while in rat cerebral arteries peroxynitrite has an inhibitory effect; the precise molecular mechanisms underlying these divergent effects are not known (251). A recent report also indicates that oxidation of SH residues activates BKCa channels in hippocampal CA1 neurons, reducing consequently the vulnerability of hippocampal neurons to hypoxia (160). Combined stimulation of BKCa channel activity by calcium and ROS, as described in Section V, should promote membrane hyperpolarization and decrease calcium entry through voltage-gated calcium channels, thus producing negative feedback for calcium signal generation. This mechanism may inhibit LTP and stimulate LTD in aging hippocampal neurons, as discussed below. Conversely, ROS-induced inhibition of BKCa channel activity should facilitate calcium entry by voltage-gated channels.

Neuronal cells express at high levels the calcium-permeable 1a subunit of acid sensing ion channels; channel activation, which generates calcium signals that are critical for synaptic plasticity and neuronal injury, is tightly regulated by channel redox state: reducing agents stimulate while oxidizing agents inhibit channel activity (76). Redox modulation of calcium permeable acid sensing channels may have important implications for normal synaptic plasticity, for neuronal function during aging and for pathological processes.

E. SERCA pump

By actively sequestering calcium into sarco-endoplasmic reticulum coupled to ATP hydrolysis, the SERCA plays a key role in maintaining a low intracellular calcium concentration. Three genes coding for SERCA1, SERCA2, and SERCA3 give rise to tissue specific isoforms by differential processing of mRNAs. Skeletal muscle expresses two SERCA isoforms: the predominant isoform of fast-twitch muscle is SERCA1, whereas slow-twitch muscle contains SERCA2, which is the only iso-

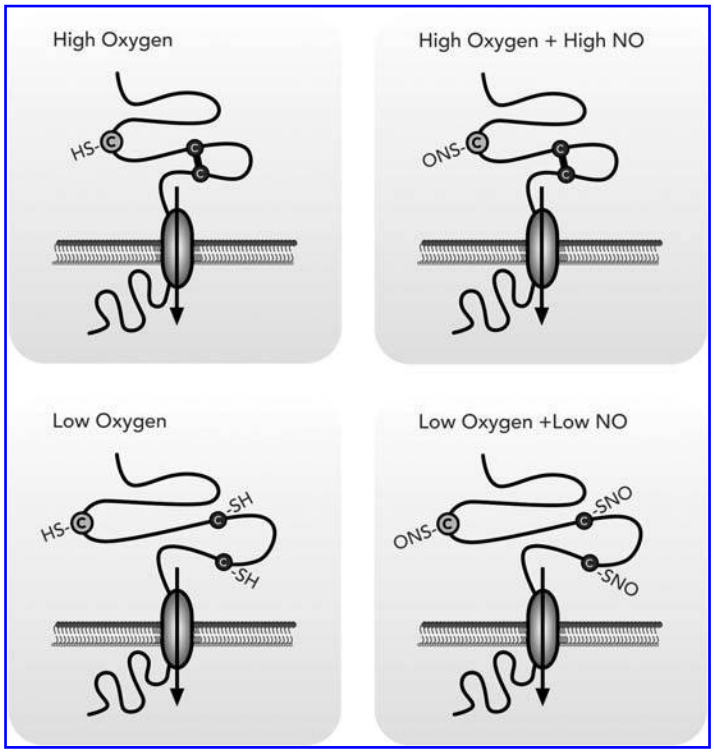

FIG. 7. A model of NO-mediated inhibition of NMDA receptor activity. According to the model illustrated in this figure, high pO_2 favors disulfide bond formation between C744 and C798 of the NMDA receptor (*upper left*). In this state, C399 can only be nitrosylated if NO levels are high (*upper right*). At lower pO_2, or under hypoxia, free thiol groups on the NMDA receptor predominate (*lower left*) making C744 and C798 available for NO-induced *S*-nitrosylation at low NO concentrations. S-nitrosylation of C744 and C798 modulate allosterically the *S*-nitrosylation of C399, resulting in channel inhibition (*lower right*). Modified from ref. 379.

form expressed in cardiac tissue. Smooth muscle expresses SERCA2b while most nonmuscle cells express SERCA3 in addition to SERCA2b (406, 426).

The different SERCA isoforms contain from 22 to 28 cysteine residues. The redox state of these residues is crucial for enzyme activity, and one or two disulfide bonds are essential for calcium transport activity (85, 205). *In vitro* studies indicate that through modification of cysteine (and tyrosine) residues ROS inhibit SERCA activity (18, 145, 431), as do peroxynitrite and NO donors (2, 148, 411–413). These isoforms differ in their susceptibility to redox inhibition (18, 145), and these differences arise presumably from the different disposition of cysteine residues in the different isoforms (51). Additionally, redox dependent interaction between SERCA2b and calreticulin, an intraluminal SR/ER protein, promotes SERCA2b activation when intrareticular calcium decreases; this regulation does not operate in SERCA2a, which lacks the 11-amino acid long COOH tail needed to interact with calreticulin (243). Incubation of muscle SR vesicles with NO donors or peroxynitrite promotes *S*-nitrosylation and *S*-glutathionylation (when incubated in the presence of GSH) of SERCA cysteine residues identified by mass spectroscopy. Incubation with a low concentration of peroxinitrite (0.1 m*M*) reversibly inactivates the enzyme and modifies 6 out of its 24 cysteine residues. Higher concentrations of peroxynitrite (0.45 m*M*) increase the number of cysteine residues modified and these modifications can be only partially reverted (412, 413). Although peroxynitrite modifies several cysteine residues, the extent of enzyme inhibition correlates with the extent of modification of a single cysteine residue (C349), which is located near the enzyme phosphorylation site (413). Yet, according to another study, this particular cysteine residue forms part of a disulfide bond and is not accessible to modification by peroxynitrite (357).

In vascular smooth muscle, NO increases SERCA-mediated calcium uptake and promotes muscle relaxation (78, 396). In the presence of GSH, low concentrations of peroxynitrite (<50 μM) induce reversible *S*-glutathionylation of the SERCA purified from vascular smooth muscle. The *S*-glutathionylation of a single cysteine residue near the hinge domain of the ATPase (C674) is responsible for enzyme activation, since a SERCA C674S mutant enzyme does not increase its activity when exposed to low peroxynitrite concentrations (2). In contrast, higher peroxynitrite concentrations (>100 μM) produce irreversible enzyme inhibition that is associated to tyrosine nitration (2). Yet, NO by itself does not promote *S*-glutathionylation of the purified SERCA, suggesting that other smooth muscle components generate from NO the peroxynitrite required to modify the SERCA.

In intact pig carotid arteries, physiological concentrations of acetylcholine and bradykinin reproduce the *in vitro* effects of peroxynitrite, suggesting that SERCA *S*-glutathionylation forms part of the physiological mechanism of arterial smooth muscle relaxation in response to endothelium-generated NO (2). The SERCA enzyme purified from rabbit aorta contains three *S*-glutathionylated cysteines in basal conditions, as revealed by MALDI-TOF MS analysis. The number of SERCA *S*-glutathionylated cysteines increases from three to seven after exposing the aorta to NO, as illustrated in Fig. 8A. Two cysteine residues are sulfonylated (HSO3-R) in the SERCA from normal aorta while nine extra cysteine residues (including C674) appear sulfonylated in the enzyme from atherosclerotic aorta (Fig. 8B). Irreversible SERCA modification in atherosclerotic aorta would prevent normal SERCA activation by NO. The irreversible modification of cysteines in atherosclerotic arteries arises from the increased ROS generation that occurs in this condition and that leads to increased peroxynitrite formation, as illustrated in Fig. 9 (2).

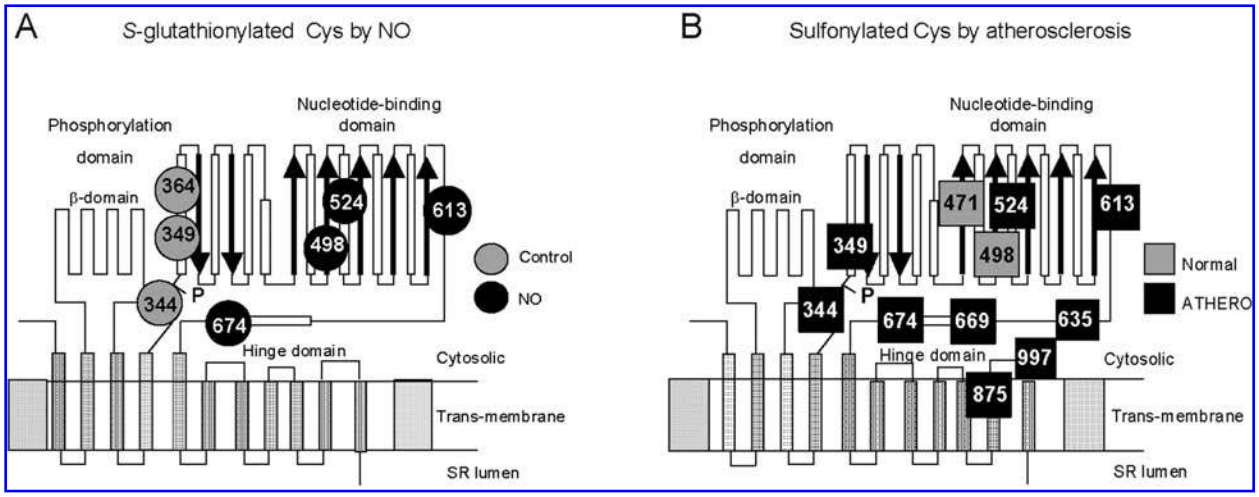

FIG. 8. MALDI-TOF MS analysis of NO-modified cysteine residues by atherosclerosis in SERCA from vascular smooth muscle. (**A**) *S*-glutathionylated cysteine residues (R-SSG) on SERCA purified from rabbit aorta under control conditions (*gray circles*) and the additional *S*-glutathionylated cysteines (R-SSG) detected after stimulation with NO (*black circles*). (**B**) Sulfonylated cysteine residues (R-SO_3H) detected on SERCA in normal aorta (*gray squares*) and additional RSO_3H detected on SERCA from atherosclerotic aorta (*black squares*). Adapted by permission from Ref. 2 (Macmillan Publishers Ltd, Nature Medicine 10, 1200, copyright 2004).

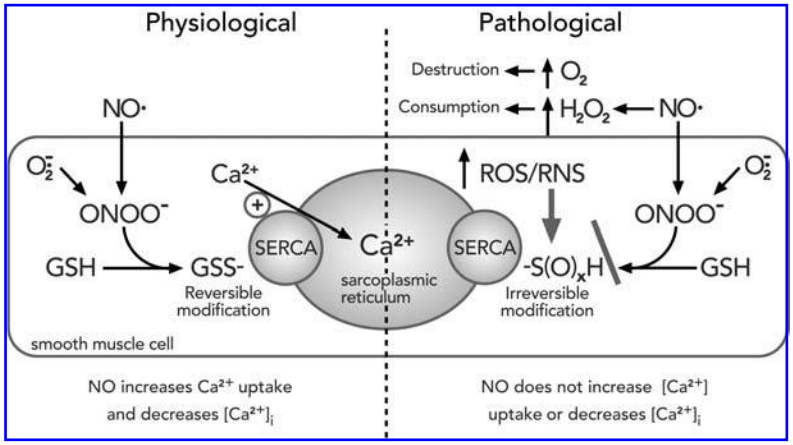

FIG. 9. Proposed mechanism of NO-induced vasodilation and the influence of disease. In intact cells and arteries, NO and superoxide generate peroxynitrite, which induces the *S*-glutathionylation of several SERCA reactive cysteines (including C674). As a result, its Ca^{2+} transport activity increases, producing smooth muscle relaxation. In pathological states, an increase in NO and superoxide, and the ROS/RNS derived from them, promote the irreversible oxidation of SERCA thiols, preventing the *S*-glutathionylation of cysteine residues critical for SERCA physiological regulation. Adapted by permission from Ref. 2 (Macmillan Publishers Ltd, Nature Medicine 10, 1200, copyright 2004).

F. IP_3R calcium release channels

The activation of phosphoinositide-specific PLC enzymes by agonist binding to plasma membrane receptors or other mechanisms promotes phosphatidylinositol 4,5-bisphosphate hydrolysis. This reaction generates diacylglycerol, which remains membrane-bound, plus the negatively charged IP_3 molecule that diffuses into the cytoplasm and binds specifically to IP_3R predominantly situated in the ER, albeit functional IP_3R are also present in the plasma membrane (90). Following IP_3 binding, the IP_3R channels open and release calcium ions stored in the ER to the cytoplasm; the resulting calcium signals play a pivotal functional role in almost every cell type (27, 191). There are three different mammalian IP_3R isoforms expressed in many cell types (386), and a number of splicing variants (34). These different IP_3R isoforms have essential roles in development and fertilization, and also play a part in exocrine secretion and synaptic plasticity in neurons (280), where different signaling microdomains regulate IP_3-mediated calcium signals (187).

In the early 1990s, several groups reported that oxidizing agents that react with protein SH residues—including endogenous redox agents such as superoxide anion and GSSG—promote IP_3R-mediated calcium release in hepatocytes (282), HeLa cells (40), cerebellar microsomes (350), and SR isolated from vascular smooth muscle (374). The stimulation of IP_3R-induced calcium release produced by oxidizing agents was attributed to increased receptor sensitivity towards IP_3 (40), so that even resting IP_3 levels would activate IP_3R modified by oxidation (282). Alternatively, the stimulation of IP_3R-mediated calcium release in bovine aortic SR was attributed to inhibition of IP_3 degradation, since superoxide failed to stimulate calcium release by a nonhydrolyzable IP_3 analog (374). Oxidized glutathione and thimerosal increase the affinity of IP_3 for its receptor in retinal cells, while DTT reverses these effects (253). Thimerosal stimulates IP_3R-mediated calcium release in platelets and reduces their GSH content (404); thimerosal also decreases the K_D of IP_3 binding to permeabilized hepatocytes and cerebellar membranes without affecting maximal binding capacity (170). These results indicate that SH reagents increase the affinity of the receptor for IP_3 essentially by decreasing the rate of IP_3 dissociation without modifying the rate of association. Increasing the Ca^{2+} concentration above 1 μM also induces a decrease in the the K_D of IP_3 binding, but in this case the rate of dissociation decreases almost three orders of magnitude while the rate of association decreases 200-fold (170).

Despite these reports, the physiological relevance of IP_3R activation by cellular ROS remained tentative until it was reported that NOX-derived H_2O_2 increases the sensitivity of intracellular calcium stores to IP_3 in human aortic endothelial cells (178). Likewise, H_2O_2 increases presynaptic activity in GABAergic interneurons via stimulation of IP_3R-mediated calcium release (378). Studies on IP_3R expressed in triple IP_3R-knockout cells derived from chicken lymphoma demonstrated that thimerosal induces redox modifications of the IP_3R1 protein (50). In addition, these authors reported that thimerosal together with calcium induce conformational changes in the N-terminal region of IP_3R1, leading to the formation of a calcium release channel highly sensitive to IP_3. These conformational changes do not occur in IP_3R3, in agreement with the lack of functional stimulation of this isoform by thimerosal, or in a mutated form of IP_3R1 lacking the N-terminal domain, strongly suggesting that they are isoform specific and involve a particular region of the IP_3R1 protein (50). Other recent reports also support direct redox modifications of the IP_3R1 protein via modification of cysteine residues present in the ER lumen. Thus, ERp44 is an ER luminal protein of the thioredoxin family that interacts directly with a luminal loop of IP_3R1 and inhibits its activity; in addition to low pH and luminal $[Ca^{2+}]$ the interaction between IP_3R1 and ERp44 requires the presence of cysteine residues with free SH groups in one of the luminal IP_3R1 loops (169, 280). Accordingly, oxidation of these IP_3R1 cysteine residues is bound to stimulate IP_3R1 activity by preventing the interaction of the calcium release channel with ERp44. In addition, a recent report indicates that 70% of the 60 total cysteine residues present per IP_3R1 monomer are kept in a reduced state in cerebellum membranes. Trypsin cleavage followed by analysis of the resulting protein fragments shows that IP_3R1 contains highly reactive cysteine residues clustered in specific regions of the molecule, which are differentially accessible to cysteine-specific probes (192).

The combined studies summarized in this section indicate that ROS modify the IP_3R1 isoform directly and stimulate calcium release mediated by this channel type. Yet, there is a clear need for further work to identify the cysteine residues respon-

sible for redox modification of IP_3R1 activity, to establish when, where, and how these modifications take place in living cells, and to determine the functional consequences of oxidative stress on IP_3R-mediated calcium release from stores.

G. RyR calcium release channels

The homotetrameric RyR calcium release channels are large integral membrane proteins (~2.3 MDa) that play crucial roles in cellular functions as diverse as muscle contraction, secretion, neuronal synaptic plasticity, fecundation, and cell death. Consistent with these important cell signaling roles, RyR activity is highly regulated by ions (H^+, Ca^{2+}, and Mg^{2+}) and small molecules (ATP, cADPR), by protein–protein interactions and by post-translational modifications including redox modifications and phosphorylation (119).

Soon after the identification of the RyR protein as the channel responsible for SR calcium release in muscle (228), several reports showed that a variety of redox agents modified RyR activity by targeting hyper-reactive RyR SH residues, as reviewed elsewhere (154, 163, 315). The first reports describing the involvement of sulfhydryl groups on RyR activity showed that heavy metals induced rapid release of calcium from skeletal and cardiac isolated SR vesicles (1, 325, 343). These early studies were followed by a several reports showing that SH reactive compounds modify RyR activity (397, 438). The subsequent identification of a discrete class of highly reactive thiols in RyR (248, 249) originated a massive amount of work that emphasized the importance of the redox state of these critical cysteine residues on RyR activity. The physiological significance of these modifications is beginning to emerge, as described below. Many reports followed these early studies, possibly making RyR the most studied protein in terms of functional modifications by ROS/RNS. It is now apparent that, in addition to exogenous redox agents, many endogenous redox compounds including NO, GSNO, hydrogen peroxide, NADH, GSH, GSSG, and varying GSH/GSSG ratios, modify RyR and significantly affect their function (164). Accordingly, RyR calcium release channels can be considered cellular redox sensor proteins (166, 314), which are susceptible to reversible *S*-nitrosylation, *S*-glutathionylation, and disulfide oxidation (7). The three mammalian RyR isoforms, RyR1, RyR2, and RyR3, are differentially present in animal tissues. Skeletal muscle contains primarily the RyR1 isoform and heart muscle the RyR2 isoform, whereas the brain and other tissues such as smooth muscle contain the RyR3 isoform in addition to varying amounts of RyR1 and RyR2 (130, 286). We will analyze next the functional effects of redox modifications of skeletal muscle RyR1, cardiac RyR2 and neuronal RyR channels.

1. Skeletal muscle. The skeletal muscle RyR1 is an essential component of the muscle excitation–contraction (E-C) coupling molecular machinery. The RyR1 isoforms are localized in specialized SR regions (the junctional SR) where they are in close physical contact with dihydropyridine receptors (DHPR), skeletal L-type calcium channels localized in plasma membrane invaginations known as transverse tubules (T-tubules) (128). Physiological activation of RyR1-mediated Ca^{2+} release in response to muscle depolarization is a very fast process (ms range) that does not require Ca^{2+} entry into the muscle cells. The depolarization of the T-tubule membrane that occurs during the active propagation of the action potential to the fiber interior induces conformational changes of the DHPR protein, which acts as a voltage sensor; DHPR depolarization-induced conformational changes promote RyR1 channel opening, which allows calcium release from the SR and muscle contraction (333).

The rabbit RyR1 isoform possesses 100 cysteine residues per monomer (381). Of these, ample evidence indicates that C3635 is functionally relevant for channel redox sensing properties (319). Based on the findings that submicromolar NO concentrations activate RyR1 at physiological (~10 mm Hg) but not at ambient (~150 mm Hg) *p*O2, the RyR1 protein was postulated as an oxygen sensor that couples channel activity to NO and pO_2 (113). Additionally, RyR1 activation by NO at physiologically low pO_2 was attributed exclusively to *S*-nitrosylation of C3635 (370), since other cysteine residues that also undergo modifications by GSNO only do so at atmospheric pO_2 (371). The proposed role of RyR1 as oxygen sensor has been questioned, however, since other authors reported that low levels of NO (0.5–50 μM) do not activate RyR1 in a wide range of pO_2 (70). Additionally, a recent report indicates that C3635 participates in E–C coupling but does not contribute to the oxidative enhancement of RyR1 activity, since H_2O_2 activates equally a C3635A-RyR1 mutant and wild-type RyR1 when expressed in human embryonic kidney cells (7).

In vitro experiments (8) indicate that *S*-glutathionylation of RyR1 cysteine residues by 5 mM GSSG decreases markedly the inhibition that Mg^{2+} exerts on RyR1-mediated CICR release rates, whereas *S*-nitrosylation with NOR-3, and NO donor, enhances RyR1 activation by Ca^{2+} but does not abolish the strong inhibitory effect of Mg^{2+} on calcium release rates (Fig. 10). These results show that *S*-glutathionylation and *S*-nitrosylation have different functional consequences, and suggest that

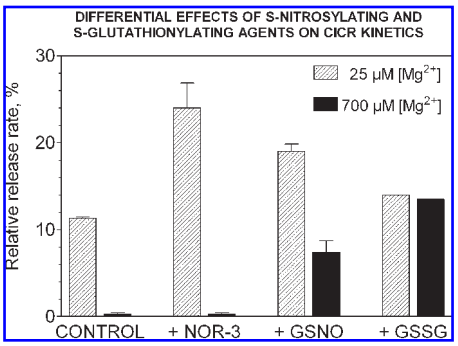

FIG. 10. Differential effects of RyR1 *S*-glutathionylation and *S*-nitrosylation on the rates of CICR. Calcium-induced calcium release kinetics from isolated SR vesicles, determined in a stopped flow spectrophotometer (8), yields initial release rates of 600 nmoles per mg of protein per s; this value corresponds to 100%. *S*-glutathionylation of RyR1 cysteine residues by 5 mM GSSG decreases markedly the Mg^{2+} inhibition of release rates. In contrast, *S*-nitrosylation with the NO donor NOR-3 (100 μM) enhances RyR1 activation by Ca^{2+} but does not abolish the strong inhibitory effect of Mg^{2+} on calcium release rates. GSNO (100 μM), which induces both cysteine *S*-glutathionylation and *S*-nitrosylation, produces a combination of both effects.

these redox modifications target different RyR1 cysteine residues. Furthermore, H_2O_2 readily induces RyR1 *S*-glutathionylation *in vitro* in the presence of GSH (9). Two large RyR1 regions (amino acids 1-2401 and 3120-4475) are major sites of both *S*-nitrosylation and *S*-glutathionylation. By using a combination of tryptic digestion, selective isotope-coded affinity tag labeling and mass spectroscopy (7), in each RyR1 subunit nine cysteine residues were found endogenously redox modified (C36, C315, C811, C906, C1591, C2326, C2363, C3193, and C3635), while three additional residues are only modified following incubation with redox agents (C253, C1040, and C1303). Each one of these cysteine residues undergoes S-nitrosylation, *S*-glutathionylation, or oxidation to disulfides, as illustrated in Fig. 11. Some of these redox-modified cysteines (C315, C811, C906, C1040, C1303) are situated in the putative FKBP12-binding site of the RyR1 N-terminal region, and some (C2326, C2363, C3635) are close to the binding sites for calmodulin and FKBP12 (7). These particular locations may explain why different RyR1 redox modifications alter calmodulin or FKBP12 binding to RyR1 (9, 370, 440). Furthermore, other residues are located within mutation regions that are associated with malignant hyperthermia (153).

These results indicate that RyR1 undergoes endogenous redox modifications in the cellular environment that may control RyR1 function in skeletal muscle cells. Thus, it becomes important to identify possible sources of endogenous ROS/RNS in these cells. Isolated SR vesicles possess a DPI-insensitive NADH dependent oxidase which co-purifies with RyR1, and generates superoxide (428). Yet, the lack of DPI inhibition and the lower activity obtained with NADPH than NADH make unlikely the identification of this NADH-dependent oxidase as a muscle Nox isoform. In addition, it was reported that the T-tubules of adult skeletal muscle fibers contain the NOX gp91^{phox} and p47^{phox} subunits, which co-localize with the DHPR α_{1s} subunit (168). *In vitro* experiments with T-tubule-containing isolated membrane fractions revealed that NADPH promotes generation of superoxide and H_2O_2, enhances RyR1 activity, and increases significantly RyR1 *S*-glutathionylation over basal levels; these effects are abolished by reducing agents or NOX inhibitors, suggesting that RyR1 channels are downstream targets of this T-tubule NOX activity (168). Since RyR1 *S*-glutathionylation decreases markedly RyR1 inhibition by Mg^{2+}, NOX-induced *S*-glutathionylation of RyR1 may increase calcium release even in the presence of the high intracellular Mg^{2+} concentrations present in intact fibers. Recent findings using field stimulation at tetanic frequencies of rat skeletal muscle myotubes in primary culture support this proposal. Upon stimulation, these cells exhibit significant ROS generation and produce large ryanodine-sensitive intracellular Ca^{2+} signals, whereas NOX inhibition with diphenylene iodonium (DPI) significantly reduces both responses (112). Hence, tetanic stimulation of skeletal muscle cells in culture enhances RyR1-mediated calcium release, presumably by causing RyR1 redox modifications by NOX-generated ROS.

Despite these findings, a definitive demonstration of the relevance of RyR1 redox modification in the context of physiological gating of the RyR1 channel during E–C coupling is still missing. Contraction of skeletal muscle fibers promotes release of superoxide anion and NO to the external medium (186), yet exogenous H_2O_2 stimulates contractions induced by caffeine but not by action potentials in skinned muscle fibers (320), while H_2O_2 activates contraction in intact skeletal muscle fibers without an visible increase in cytoplasmic Ca^{2+} concentration (6). These combined results suggest that ROS do not have a role in physiological E-C coupling; yet, a direct demonstration that H_2O_2 addition to skeletal muscle cells modifies RyR1 redox state has not been provided. This is an important issue to consider, because antioxidant defense systems may readily scavenge exogenously added H_2O_2 before it reaches RyR1 at the triads. The existence of efficient cellular antioxidant defenses highlights the relevance of ROS source location. It was recently reported that superoxide anion production by the skeletal muscle T-tubule NOX represents a minor fraction when compared to mitochondrial superoxide anion generation (403). Yet, generation of superoxide anion by the T-tubule NOX in the immediate vicinity of RyR1 represents a clear advantage compared to its generation by mitochondria, which are at some distance from RyR1 and separated from them by the SR lumen (127). Moreover, superoxide anion and H_2O_2 generated by the NOX enzyme present in T-tubules may escape the scavenging effects of endogenous antioxidant systems, allowing RyR1 modification in the highly restricted space between junctional

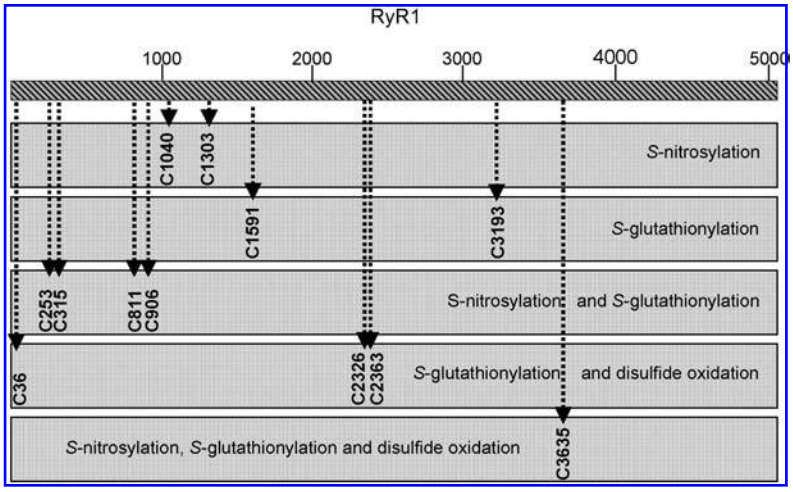

FIG. 11. Location of RyR1 cysteines modified by *S*-glutathionylation, *S*-nitrosylation, and disulfide oxidation. Nine cysteine residues in each RyR1 subunit were found endogenously redox modified (C36, C315, C811, C906, C1591, C2326, C2363, C3193, and C3635), whereas three additional residues are only modified following incubation with redox agents (C253, C1040, and C1303). Each one of these cysteine residues undergoes S-nitrosylation, *S*-glutathionylation. or oxidation to disulfides (7), as illustrated in the figure.

SR and T-tubules. The privileged location of the skeletal muscle NOX at the T-tubules and its activation by tetanic stimulation open the possibility that conditions of increased muscle activity, such as exercise, stimulate Ca^{2+} release through NOX-induced RyR1 redox activation. Furthermore, H_2O_2 and NOS inhibitors modify the voltage dependence of calcium release activation during E-C coupling in intact muscle fibers; these changes most likely reflect RyR1 redox-related modifications since changes in DHPR were ruled out (322).

2. Cardiac muscle. The RyR2 isoform contains 89 cysteine residues per monomer. Of these, a few are hyper-reactive and are rapidly oxidized by ROS and RNS, including hydrogen peroxide, superoxide anion, and nitric oxide. Nonphysiological SH reagents, as well as endogenous ROS/RNS, increase the opening probability of cardiac RyR2 channels in lipid bilayers, and calcium release fluxes from isolated cardiac SR vesicles (163, 164). Additionally, short exposure to H_2O_2 enhances calcium transients generated by field stimulation of isolated cardiomyocytes, albeit these effects may not be due exclusively to redox-induced stimulation of RyR2 activity (139, 373). In contrast, intracellular dialysis of cardiomyocytes with DTT greatly reduces calcium transients, despite the small increase in the inward calcium current observed in the presence of DTT (139, 373).

The identification of the cysteine residues modified in RyR2, or the type of redox modification they undergo, have been scarcely addressed since most studies have characterized the effects of redox active compounds without investigating further the covalent modifications of the channel molecule. There is only one report (432) showing that incubation of purified RyR2 with different NO donors promotes *S*-nitrosylation of up to three cysteine residues per monomer, with a stepwise increase in RyR2 activity. Furthermore, at high concentrations, some NO donors activate the channels by irreversible oxidation of seven or more thiols per subunit (432).

There are few studies on the effects of ROS/RNS on RyR2 function under physiological conditions. The RyR2 isoform is *S*-nitrosylated (432) and *S*-glutathionylated (345) *in vivo*. The low level of RyR2 *S*-nitrosylation observed under basal conditions can increase on a millisecond time scale, leading to channel activation. The neuronal NOS isoform, which localizes to the SR membrane, may control calcium cycling and exert a positive inotropic effect. Yet excessive generation of NO can produce extensive RyR2 *S*-nitrosylation or oxidation of multiple cysteine residues leading to irreversible activation of the channel with the ensuing loss of control of SR calcium content (272, 446). In addition, RyR2 *S*-glutathionylation increases after increasing heart rate by electrical stimulation (tachycardia) and the increase in *S*-glutationylation correlates with an increase in calcium release rate in isolated vesicles (Fig. 12); exercise produces similar effects (344). Incubation of isolated SR vesicles with NADPH in the presence of trace amounts of GSH increases

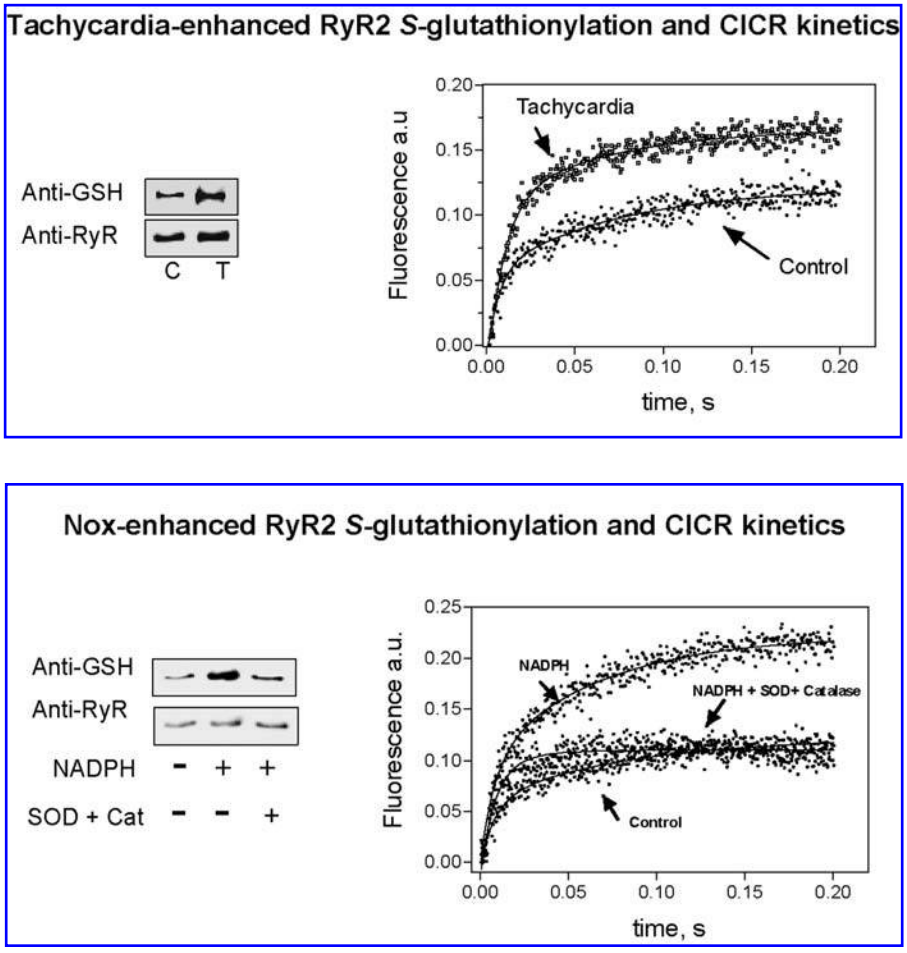

FIG. 12. RyR2 *S*-glutathionylation modifies CICR in isolated SR vesicles. *Upper panel, left*: representative Western blots obtained in SR vesicles from control (C) or after electrically induced tachycardia (T) probed with anti-GSH or anti-RyR antibodies. *Upper panel, right*: representative fluorescent records illustrating CICR kinetics in SR vesicles obtained from control or after tachycardia. SR vesicles actively loaded with Ca^{2+} were mixed (1:10) in a stopped flow fluorescence spectrometer with a solution that produced upon mixing pCa 6 and 1.2 m*M* free ATP. Release kinetics were measured following the change in fluorescence of Calcium Green 5 N. *Lower panel left*: representative Western blots obtained in isolated SR vesicles from control (C) or after incubation with NADPH in the presence or in the absence of SOD plus catalase, probed with anti-GSH or anti-RyR antibodies. *Lower panel right*: calcium release kinetics in SR vesicles after incubation with NADPH in the presence or in the absence of SOD plus catalase. Release kinetics were determined as above. Experimental data are from ref. 345.

both RyR2 *S*-glutathionylation and calcium release, mimicking the effect of tachycardia. Incubation of SR vesicles with SOD plus catalase prevents both RyR2 *S*-glutathionylation and the increase in calcium release kinetics induced by NOX stimulation with NADPH. These results suggest that tachycardia and exercise increase NADPH oxidase activity, which enhances RyR2 activity by promoting RyR2 *S*-glutathionylation (344, 345). The precise mechanisms by which *S*-glutathionylation of RyR2 increases calcium release activity remain unknown. Among other possibilities, redox enhancement of RyR2 activity might be caused by changes in the binding of calmodulin, which inhibits the channel, or of FKBP12.6, which stabilizes the channel, since binding of both proteins to RyR2 is redox sensitive (17) (447).

3. Neurons. Several neuronal functions, including synaptic plasticity and gene expression, require transient elevations of $[Ca^{2+}]i$ (28, 30). Evidence reported in the last few years indicates that both ROS and RNS have an effect on neuronal calcium signals. Hydrogen peroxide elicits calcium signals in hippocampal neurons in culture (208), while iron-generated ROS do so in PC12 cells (165). Generation of NO either inhibits (75, 176) or potentiates (177, 206) neuronal calcium signals. The magnitude and duration of post-synaptic calcium signals determine dendrite growth or retraction and markedly influence activity-dependent synaptic plasticity (422). A brief, large magnitude rise in postsynaptic $[Ca^{2+}]i$ elicits long-term potentiation (LTP), an increase in synaptic transmission that may persist for hours or days. Conversely, long-term depression (LTD) is a decrease in synaptic strength induced by a modest and prolonged rise in $[Ca^{2+}]i$. RyR-mediated Ca^{2+} release may contribute to the generation of the calcium signals required for synaptic plasticity and gene expression in neurons (61), and may also be involved in causing neurodegenerative processes, as discussed in Section V. Of the three RyR isoforms expressed in different brain regions, RyR2 is the most abundant (130, 133, 286). Yet, in spite of their emerging importance for neuronal function, few reports exist on the redox regulation of the RyR calcium release channels present in brain.

The activation by Ca^{2+} and ATP of single RyR channels from rat brain cortex incorporated in planar lipid bilayers depends on the redox state of the channels (47, 48, 264). In brief, highly reduced single channels incorporated in bilayers respond poorly to elevations of *cis* (cytoplasmic) $[Ca^{2+}]$, whereas increasing cysteine oxidation/alkylation enhances channel activation by Ca^{2+} even in the presence of high $[Mg^{2+}]$. This behavior of RyR single channels from brain cortex ER incorporated in lipid bilayers is illustrated in Fig. 13. As described above, hydrogen peroxide stimulates RyR *S*-glutathionylation and elicits RyR-mediated calcium signals in hippocampal neurons in culture (208), whereas iron-generated ROS promote the emergence of RyR-mediated calcium signals in PC12 cells (165). Furthermore, glucosylceramide, a glycosphingolipid implicated in neuronal degeneration, also enhances RyR-mediated calcium release from rat brain microsomes (252).

Several studies indicate that the strong neuronal stimulation required for sustained LTP in the hippocampus promotes the generation of ROS, that are required for LTP (217). Other reports indicate that RyR-mediated Ca^{2+} release is also needed

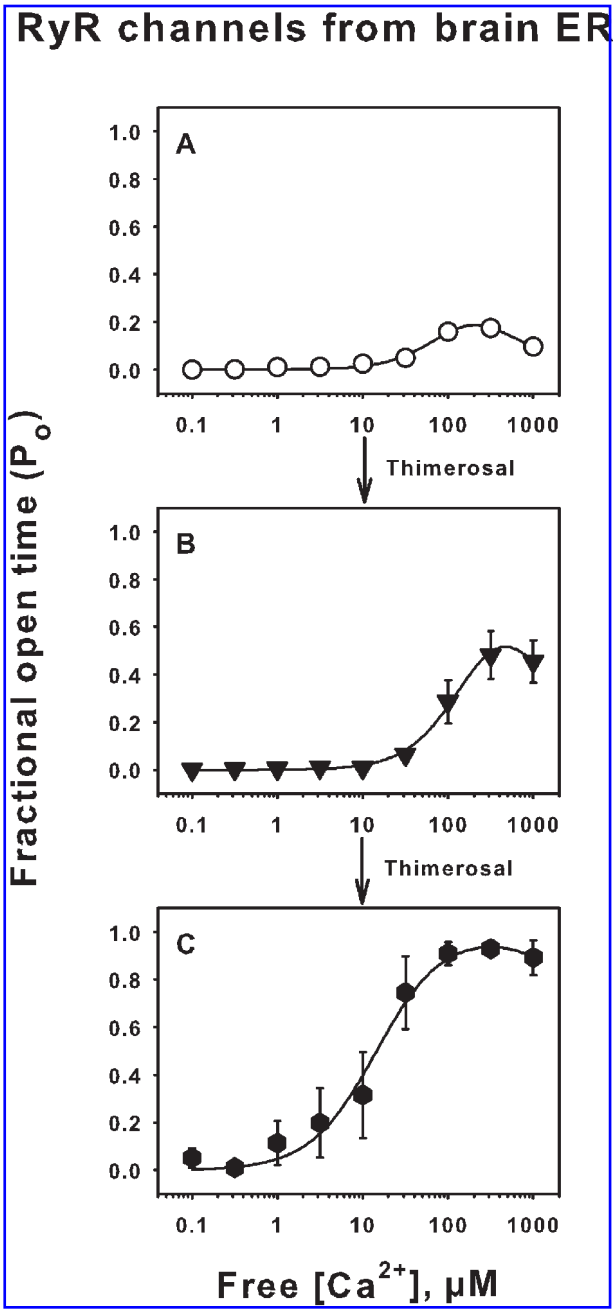

FIG. 13. Sequential increase in RyR single channel activity after increasing cysteine oxidation/alkylation by incubation with thimerosal. Calcium dependencies of control and redox-modified RyR single channels. The figure illustrates the mean fractional open times (P_o) as a function of free cytoplasmic (cis) $[Ca^{2+}]$ in the presence 0.8 m*M* free $[Mg^{2+}]$ and 0.5 m*M* free [ATP] of single RyR channels from brain cortex. (**A**) Control (highly reduced) channels are scarcely activated by $[Ca^{2+}] > 30\ \mu M$. (**B**) Control channels incubated with thimerosal exhibit a higher increase in activity than control channels, but only at $[Ca^{2+}] > 30\ \mu M$. (**C**) Further incubation with thimerosal of the channels shown in **B** promotes significant channel activation by $[Ca^{2+}] > 1\ \mu M$. Channels exhibit maximal activity at $[Ca^{2+}] \geq 100\ \mu M$. Modified from ref. 47.

for hippocampal LTP induction (165). It is possible to speculate that the concomitant increase in postsynaptic $[Ca^{2+}]_i$ and ROS/RNS that takes place during sustained LTP induction may enhance RyR-mediated Ca^{2+} release, producing the strong Ca^{2+} signals required for ERK/CREB stimulation needed for long-lasting hippocampal LTP (61). As mentioned above, ROS promote RyR-dependent Ca^{2+} release in hippocampal neurons in primary culture (62, 208, 209). ROS also activate CREB and ERK phosphorylation and immediate early genes, while iron-generated ROS promote ERK phosphorylation via RyR-dependent Ca^{2+} release (165). These results suggest that oxidative stimulation of RyR-mediated Ca^{2+} release might be an intermediate step in the activation of the ERK/CREB phosphorylation cascade required for sustained synaptic plasticity.

The results discussed above suggest that activation of RyR channels by cellular ROS/RNS may represent a physiological mechanism of crosstalk between Ca^{2+} and redox signaling pathways. Thus, cells may use redox-modulated RyR-mediated Ca^{2+} release as an additional mechanism to either amplify or inhibit Ca^{2+} signals as needed for a specific response. In neurons, generation of ROS/RNS such as hydrogen peroxide or NO, which may act as diffusible signal molecules in synaptic plasticity, may modify cellular processes that depend on RyR-mediated Ca^{2+} release from the ER, including long-term potentiation and long-term depression, and presumably learning and memory. Moreover, oxidative stress and alterations in Ca^{2+} homeostasis may contribute to neuronal apoptosis and excitotoxicity, which may underlie the pathogenesis of several neurodegenerative disorders. In particular, RyR channels may be involved in the pathophysiology of neurodegeneration in Alzheimer's disease (207, 288).

H. Calcium entry and release pathways in other organelles

In addition to the ER, other intracellular compartments, chiefly the mitochondria, the nucleus, and the Golgi apparatus, possess pathways that allow them to take up and release calcium (324). Albeit there is no information on the direct regulation of these pathways by ROS/RNS, a brief description of the contribution of these organelles to calcium signaling follows.

1. Mitochondria. This organelle makes an important contribution to cellular energy production, redox status, calcium signaling, and programmed cell death. Knowledge on the participation of mitochondria on cellular calcium signaling has increased significantly in the last years (336). It is now apparent that mitochondrial calcium uptake and release not only influence cellular calcium signals and ROS generation but also determine mitochondrial function and integrity (45). The presence of a large capacity entry pathway plus the steep electrochemical gradient for calcium ions and the large calcium binding capacity of the mitochondrial matrix allow mitochondria to effectively take up calcium when $[Ca^{2+}]$i reaches threshold levels (297, 335). Entry of calcium ions into mitochondria, which occurs mainly through the calcium uniporter, gives rise to mitochondrial calcium signals in multiple cell types (55). The mitochondrial uniporter is a highly selective calcium channel (216) that mediates calcium entry into the mitochondria utilizing the driving force provided by the mitochondrial membrane potential. Extensive mitochondrial calcium uptake, however, dissipates this membrane potential and interferes with mitochondrial function, and may prompt the opening of the mitochondrial transition pore. Calcium ions move out of mitochondria through a Na^+/Ca^{2+} exchanger and through a Na^+-independent system, although the calcium uniporter and the permeability transition pore may also act as calcium efflux routes (55). Calcium signals can activate both the calcium uniporter and the VDAC component of the permeability transition pore, this activation is relevant for the homeostatic control of cytoplasmic $[Ca^{2+}]$i (19). There are no reports, however, of direct redox modulation of the uniporter or the mitochondrial NCX by intramitochondrial or cytoplasmic ROS/RNS. Mitochondria partially buffer calcium signals but also influence signal propagation; they can operate either as a barrier buffer or as a facilitating factor in the spreading of calcium signals, effectively modifying calcium signals elicited by IP_3R and RyR (4). In addition, there is a highly coordinated crosstalk between mitochondrial and nuclear calcium signals (326); through this mechanism mitochondria control specific nuclear responses that can greatly influence cell behavior.

2. Nucleus. Nuclear calcium signals control gene transcription and DNA synthesis and repair, and thus have a key role in cell signaling and survival. Calcium signals can arise at the nucleus from propagated cytoplasmic calcium signals or by other mechanisms, including calcium release directly at the nucleus (60, 105). Putative calcium stores in the nucleus include the nuclear envelope and its invaginations inside the nucleoplasm that make up the nucleoplasmic reticulum (262). There is also evidence for mitochondrial involvement in the generation of calcium signals from nuclear calcium stores (255). The nuclear envelope can also release calcium into the nucleus via IP_3R or RyR (4, 266), both of which are highly redox-sensitive proteins. Hence, calcium release mediated by these channels is likely to respond to physiological and pathological changes in nuclear redox state, albeit no information is currently available in this regard.

3. Golgi. The Golgi apparatus, which is involved in sorting and processing secretory and membrane proteins, can function as a calcium store (427), releasing calcium rapidly following cell stimulation with an agonist that promotes IP_3 generation (93, 318). Changes in $[Ca^{2+}]$ in the cytoplasm or in the Golgi lumen regulate the function of this organelle, whereas the $[Ca^{2+}]$ within its lumen controls protein processing and sorting. Despite the fact that disruption of the Golgi apparatus or release of calcium from the Golgi have significant effects on intracellular calcium signals (409), there are no reports on direct effects of ROS/RNS on calcium uptake or release from the Golgi. Since SERCA isoforms mediate in part calcium uptake into the Golgi apparatus (408), redox modulation of the function of this protein in the Golgi is to be expected.

I. Other calcium-sensing proteins

In addition to the proteins already mentioned, calcium and redox signals jointly modify the function of proteins involved in cellular signal transduction pathways—including small G

proteins, transcription factors, and enzymes—and of calcium-binding proteins and ion channels. Yet, as pointed out recently (81), the functional effects of cysteine modifications in the response of these proteins to cellular physiologic stimuli has proven more difficult to demonstrate.

1. cAMP signaling proteins. The nine isoforms of the mammalian adenylyl cyclase (AC1–AC9) that have been cloned have a unique pattern of expression and are distinguished by their distinctive regulatory properties, which presumably underlie their physiological roles (155, 218). Only some AC isoforms are calcium sensitive. Calcium/calmodulin activate AC1 and AC8 in the central nervous system and calcium at sub μM concentrations exerts an inhibitory effect on AC5 and AC6, while AC9 is the only isoform that is potentially inhibited by the calcium-dependent phosphatase calcineurin (155, 218). Both ROS and RNS promote the oxidation of critical cysteine residues of type I adenylyl cyclase, resulting in loss of calmodulin sensitivity (103). Furthermore, in addition to its well-known effects on soluble guanylyl cyclase, NO modulates cAMP signaling by targeting AC5 and AC6 (218), while ROS-induced *S*-glutathionylation of cAMP-dependent protein kinase (cAPK, PKA) also regulates cAMP signaling (221). These findings indicate that some AC isoforms are dual targets of calcium and ROS/RNS.

2. Small GTPases. Numerous observations suggest that the Ras superfamily of small GTPases have a significant role in redox regulation, and there is some evidence that ROS serve as important downstream effectors for both Ras and Rac (122). Ras activation stimulates the MAP kinase ERK1/2 cascade, which participates in many cellular functions and plays a central role in neuronal synaptic plasticity, as discussed above. Of relevance in the context of this review is the joint modulation of Ras by calcium (82) and ROS (122).

3. Kinases and phosphatases. Calcium signals also stimulate directly or via Ca^{2+}-CaM the activity of several kinases and phosphatases involved in signal transduction, including calcineurin, tyrosine protein phosphatases, CaM kinases, PKA, calcium-dependent protein kinase C (PKC) isoforms, and lipid kinases. All these enzymes are targets of ROS/RNS and also phosphorylate or dephosphorylate several redox sensitive proteins involved in calcium homeostasis and signaling, that are thus subject to direct and indirect regulation by ROS/RNS (132). For instance, superoxide anion inhibits calcineurin, the Ca^{2+}- and CaM-dependent serine/threonine protein phosphatase that modulates the activity of a number of Ca^{2+}-dependent signal transduction pathways and transcription factors (342). Superoxide inhibits calcineurin activity by interacting with its Fe–Zn active redox center (141), whereas SOD prevents calcineurin inactivation both *in vitro* and *in vivo* (416). The opposite effects of calcium and ROS on its activity make calcineurin an interesting cellular target for fine-tuning the activity of proteins phosphorylated by serine/threonine kinases. Thus, if calcium stimulation of calcineurin prevails over ROS inhibition, dephosphorylation of its protein substrates will be stimulated, while the opposite balance will prolong their phosphorylated status. Section V addresses the possible role of calcineurin as a joint target of ROS and calcium during aging and neuronal degeneration.

Whereas ROS seem to both activate or inhibit CaM kinases (126), NO promotes the reversible inactivation of PKC activity (140). Yet the two domains of PKC respond differently to redox agents: oxidants selectively react with the regulatory domain and stimulate PKC, whereas antioxidants react with the catalytic domain and inhibit PKC activity (141). In addition, redox modification of PKC delta, a redox-sensitive member of the novel PKC family, may have a role in promoting cellular apoptosis (200).

ROS/RNS also affect the activity of tyrosine kinases and phosphatases and of some enzymes involved in phosphoinositide metabolism, including the phosphatidyl inositol 3-kinase signaling cascade (236). For example, H_2O_2 appears to promote protein tyrosine phosphorylation by activating protein tyrosine kinases such as Src (134), while oxidation of a conserved catalytic cysteine residue inactivates protein tyrosine phosphatases (425). Likewise, in cultured astrocytes and brain slices, oxidative stress induced by hydrogen peroxide elicits inositol phosphate accumulation via enhanced PLC-mediated phospholipid breakdown (356). Consequently, cellular ROS generation can also indirectly affect calcium signaling through stimulation or inhibition of tyrosine kinases and phosphatases and of enzymes that act on phosphoinositides engaged in calcium signaling.

4. Gene expression and transcription factors. Besides kinases and phosphatases, ROS/RNS and calcium regulate gene expression. Calcium-dependent gene expression, which involves signal transduction pathways that engage protein phosphorylation cascades as well as cytoplasmic and nuclear calcium signals, is central to many cellular responses. Likewise, redox-based regulation of gene expression is a fundamental cellular regulatory mechanism (355). Protein phosphorylation and dephosphorylation by tyrosine and serine/threonine kinases and phosphatases is redox sensitive; so are many of the molecules responsible for the generation and regulation of calcium signals that in turn affect protein phosphorylation and gene expression. For this reason, their interactions and redox modulation are highly complex and probably depend on their temporal properties as well as the cellular location of ROS/RNS and calcium sources.

Calcium signals and ROS regulate the activity of a variety of transcription factors including the cAMP/calcium response element binding protein (CREB), the nuclear factor of activated T cells (NFAT) and NF-κB. Several reviews have addressed this issue in recent years (42, 61, 62, 161, 257, 274, 323).

5. Calcium-binding proteins. Cytoplasmic calcium-binding proteins have a central role as buffers and targets of calcium signals (29). Redox modulation of calmodulin (349) was addressed above. Yet to our knowledge, there are no published studies on the effects of ROS/RNS on other calcium binding proteins, such as calretinin, parvalbumin, troponin C, synaptotagmin, and the annexins. We will focus on the redox modulation of calcium-binding proteins for which information is available: calbindin, the S-100 proteins, neurogranin, and neuromodulin. Calbindin is a calcium-binding protein implicated in cell protection against apoptosis and differentiation of fetal and adult hippocampal cells in culture (211). Human cal-

bindin D(28k) has two N-terminal cysteine residues that undergo redox-driven structural changes consistent with disulfide bond formation, whereas the three C-terminal cysteine residues can be modified by glutathione. The redox state of calbindin D(28k) cysteine residues seems to be functionally relevant, since the oxidized protein binds calcium ions with lower affinity than the reduced protein, and the two N-terminal cysteine residues are required for activation of myo-inositol monophosphatase; moreover, enzyme activation is enhanced under conditions that promote oxidation of these residues (66).

The small and acidic calcium-binding proteins of the S100 family are involved in the control of a number of cellular processes. They undergo redox modifications activated by calcium binding, including *S*-nitrosylation and *S*-glutathionylation. Yet, understanding how S100 proteins participate in the control of redox-based signal transduction awaits further studies (137, 275, 445). The calcium-binding proteins neuromodulin and neurogranin present in neuronal synaptic terminals are also subject to redox modulation (240). Changes in neurogranin redox state and phosphorylation modify its binding to CaM and the subsequent signaling elicited by CaM-activated CaM kinase II in postsynaptic terminals (180, 424, 429).

V. CELLULAR OXIDATIVE STRESS AND PATHOLOGICAL CONDITIONS

In summary, the results described above in Sections III and IV indicate that many cellular proteins participate in the crosstalk that takes place between calcium and ROS/RNS signaling, as illustrated in the scheme presented in Fig. 14. Whereas these previous sections dealt essentially with the physiological aspects of this crosstalk, Section V will address the implications of cellular oxidative and nitrosative stress for cell function.

A proper balance between ROS/RNS production and cellular antioxidant capacity sets the cellular redox tone and keeps it within physiological limits. Consequently, oxidative or nitrosative stress arises when excessive ROS/RNS production overcomes the cellular antioxidant defense mechanisms. For simplicity, we will use the general term oxidative stress to encompass both oxidative and nitrosative stress. Oxidative stress perturbs the function of many cellular processes. Among other harmful effects, oxidative stress causes glutathione deficiency (423) and promotes cell death by inducing mitochondrial depolarization, membrane lipid peroxidation, and DNA damage, among other harmful effects. To counteract the damaging effects of excess ROS/RNS, cells make use of enzymatic and nonenzymatic defense mechanisms, some of which also engage calcium signals (142). Dependent on the extent and persistence of the cellular damage, oxidative stress may play a key role in the pathogenesis of diverse diseases, including Alzheimer's and Parkinson's disease, stroke, diabetes, and cardiac pathologies (104, 144, 152, 284, 338).

A common feature of pathological states associated with oxidative stress is an increase in $[Ca^{2+}]i$, which if not properly controlled can induce apoptotic or necrotic cell death through cellular calcium overload (303). Contingent on cell type and cell age, oxidative stress may increase $[Ca^{2+}]i$ by inducing a failure of calcium homeostatic mechanisms and/or through stimulation of channels engaged in cellular calcium influx and release from intracellular stores. To cite just a couple of examples, ROS and a disruption of intracellular calcium homeo-

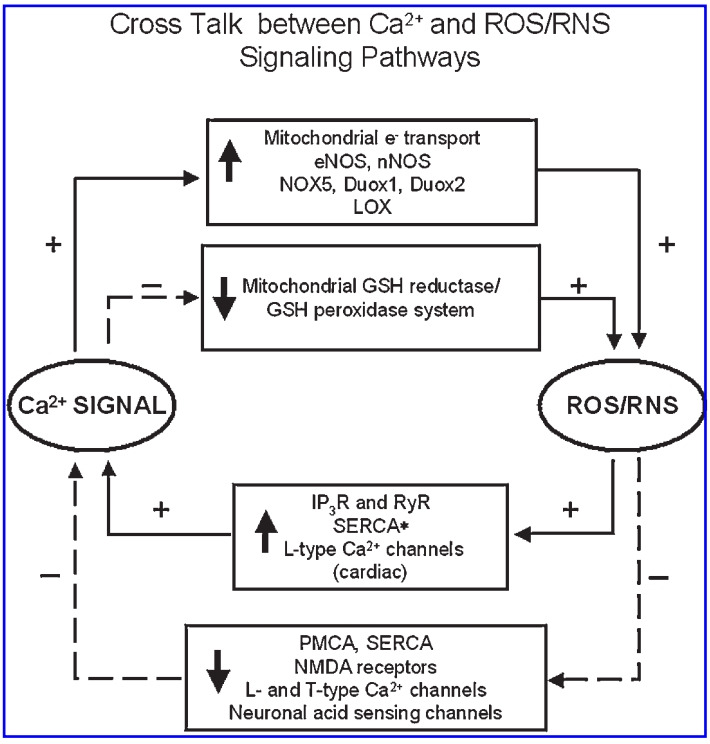

FIG. 14. Simplified scheme illustrating crosstalk between Ca^{2+} and ROS/RNS signaling. Calcium signals, defined as transient increases in intracellular free calcium concentration, activate several enzyme systems that generate ROS and RNS. The resulting activation or inhibition of various calcium channels and transporters by ROS/RNS modify in turn the original calcium signal. *In aortic smooth muscle, the SERCA is activated by RNS (see Fig. 8).

stasis play a part in the earliest stages of acute pancreatitis (77), whereas oxidative stress promotes the formation of 4-hydroxy-2,3-nonenal (HNE), a membrane lipid peroxidation product that by enhancing calcium influx through NMDA receptors may have a role in physiological and pathophysiological responses of neurons to oxidative stress (254).

A. Oxidative stress, mitochondria, ER, and cell death

Oxidative stress can modulate apoptotic cell death by reducing overall cellular function as a consequence of a general increase in oxidized proteins, or via specific modifications of signaling proteins—including some proteins involved in calcium signaling—with the ensuing deregulation of many cellular signaling pathways (110). In particular, an uncontrolled increase in $[Ca^{2+}]i$ produced by abnormal calcium signals, plus emptying of intracellular calcium stores provokes apoptotic or necrotic cell death in almost all cell types (303).

The importance of mitochondria in the life and death of a cell is a current subject of intense study (195). Mitochondria constantly generate superoxide anion through reaction of unpaired electrons that escape from the electron transport chain with molecular oxygen. Mitochondrial Mn-SOD converts superoxide anion to hydrogen peroxide, that by being readily permeable escapes to the cytoplasm. Hence, a prolonged increase in mitochondrial activity is likely to cause cellular oxidative stress (15). In addition, mitochondria-generated superoxide and hydrogen peroxide react with NO to produce RNS, which stimulate further superoxide, hydrogen peroxide, and peroxynitrite mitochondrial production; excessive ROS/RNS generation inhibits mitochondrial respiration and can induce cell death by apoptosis or necrosis (46). Many groups have shown that mitochondria take up significant amounts of calcium under pathological conditions of cytoplasmic calcium overload (101, 102, 347). The resulting mitochondrial calcium overload reduces mitochondrial membrane potential and accelerates electron transport, which enhances mitochondrial ROS production and thus induces further cellular stress that may lead to cell death (235, 296). The mitochondria permeability transition pore and the ER/SR are potential cellular targets of oxidative stress (151). Opening of the mitochondrial permeability transition pore can lead to mitochondrial swelling and release of cytochrome C and other proapoptotic factors, resulting in cell death by apoptosis or necrosis (223). Thus, a cell death program may proceed if large calcium signals produced by strong stimulation of ER/SR calcium release channels propagate to neighboring mitochondria and cause the permeability transition pore to open; moderate calcium signals may produce the same effect if they coincide with a sensitized permeability transition pore (19). In this context, evidence is emerging that calcium transfer from the ER/SR to the mitochondria as well as mitochondrial morphology may decide if the final outcome will be cell survival or cell death (376). In addition, conditions that promote calcium entry and cause mitochondrial $[Ca^{2+}]$ to increase moderately inhibit the mitochondrial glutathione reductase/glutathione peroxidase system that removes hydrogen peroxide (448). This inhibition may further promote mitochondrial redox imbalance.

Diverse defense mechanisms protect mitochondria from oxidative damage. Superoxide anion may activate mitochondrial uncoupling proteins at the matrix side of the mitochondrial inner membrane; the enhanced proton transport may limit further mitochondrial ROS production by decreasing mitochondrial membrane potential (12, 106). Yet, the *in vivo* relevance of this mechanism remains to be established (33). The transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a key regulator of mitochondrial biogenesis and respiration, represents another possible mechanism of mitochondrial protection; it increases mitochondrial electron transport activity and stimulates in parallel cellular defense systems that reduce oxidative stress. Furthermore, in endothelial cells NO regulates mitochondrial oxidative stress, and the ensuing expression of genes involved in oxidative protection, through early downregulation followed by secondary late upregulation of PGC-1α expression (41).

Together with the mitochondria, the ER plays a major role in the control of cell death, and evidence is beginning to emerge that calcium signals plus oxidative stress have marked effects on ER function (143). Current available evidence indicates that a number of neurological diseases are associated with ER perturbations, including abnormal changes in luminal calcium content. Glycosphingolipids, including gangliosides that are present at the ER membrane, regulate the ER calcium concentration (84). Noteworthy, accumulation of glucosylceramide, which is the main storage product in Gaucher disease, increases ER calcium release in cultured neurons presumably via amplification of the response to agonists of the highly redox sensitive RyR protein (252).

In addition to its well-established function as calcium store, the ER regulates correct protein folding, which requires the oxidative environment of the ER lumen for intramolecular disulfide bond formation in proteins via protein-disulfide isomerase (PDI). Moreover, many ER chaperones require defined calcium concentrations to assist efficient protein folding, so that calcium depletion from the ER leads to accumulation of misfolded proteins. Under stress conditions, the ER generates the unfolded protein response—a complex stress response that promotes cell survival (441). The ER stress response makes use of a variety of mechanisms to limit, if possible, the harmful effects of unfolded protein accumulation (353). It follows that abnormal protein folding due to changes in ER redox state combined with ER calcium depletion may lead to abnormal cell function and even cell death. Furthermore, oxidative stress conditions may induce cell death through uncontrolled activation of the redox-sensitive ER/SR calcium release channels (418), and the ensuing ER calcium depletion (261, 420). Further evidence in this regard comes from studies in ischemic rat brain showing that dantrolene, a RyR antagonist, decreases the ER stress response induced by ischemia and the ensuing activation of apoptotic signaling pathways (239). Additionally, recent studies suggest that ER stress has a role in Parkinson's disease (415) as described in further detail when addressing this subject below.

Calcium signals can promote NO generation (see Section III, B) and evidence has been provided that NO-dependent *S*-nitrosylation of protein cysteine residues regulates apoptosis (263). Contingent on cell type and cellular redox state, NO has a dual role as pro- or antiapoptotic agent (73). The recently identified NO-induced *S*-nitrosylation of Bcl-2, a redox-sensitive antiapoptotic protein, represents an example of the capacity of NO to act as antiapoptotic agent; multiple apoptotic me-

diators and stress conditions promote *S*-nitrosylation of two specific Bcl-2 cysteine residues (Cys158 and Cys229) and prevent its downregulation via the ubiquitin–proteasome pathway (14). Likewise, NO inhibits the formation of the apoptosome complex, an essential step of caspase-mediated apoptosis (439). In contrast to its antiapoptotic effects, NO also activates, via several possible pathways, a complex network of responses leading to apoptosis. Thus, NO generation prompts *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase, which in turn triggers nuclear signaling cascades that promote apoptotic cell death (156). Via *S*-nitrosylation of protein-disulfide isomerase, NO may also contribute to the accumulation of misfolded proteins in some neurodegenerative conditions (400). Furthermore, in cerebellar granule cells GSNO promotes ER calcium depletion, with a sustained increase in $[Ca^{2+}]i$, depletes intracellular GSH, and produces ER stress combined with upregulation of proapoptotic genes and downregulation of antiapoptotic genes (158).

B. Age-related oxidative stress and calcium signaling

A vast literature indicates that cellular oxidative stress increases during biological aging (15). The reduction in PMCA activity produced by oxidation may be responsible for the increased resting $[Ca^{2+}]$ observed in neurons of aged brains. Thus, a decrease in the capacity to maintain resting intracellular $[Ca^{2+}]$ within normal limits may contribute to the cellular damage observed in age-related pathologies (366). Additionally, reversible oxidation of CaM methionine residues gives rise to their corresponding methionine sulfoxides during normal biological aging; hence CaM redox modulation may play a regulatory role in coupling calcium signaling to cellular redox conditions during aging (367). Age-related oxidative stress is also bound to alter cellular calcium homeostasis as a consequence of the significant loss of SERCA activity due to oxidation of cysteine and nitration of tyrosine residues (367). Mass spectroscopy analysis of the SERCA isoform present in fast skeletal muscle indicates that in aged animals ten cysteine residues are partially modified *in vivo*. Yet, the cysteine(s) responsible for the ensuing loss of activity remain unidentified. Furthermore, the labeling technique used to analyze cysteine modifications cannot specify the nature of their oxidation products (357). The ER resident protein disulfide isomerase Grp58—also known as ER-60/ERp57—alters SERCA activity via modification of some of its cysteine residues (243), which may vary during aging. Harmful modifications of the redox sensitive IP_3R and RyR calcium release channels may also occur as a consequence of age-related oxidative stress, promoting calcium leak from intracellular stores (377).

Aging brings about particularly harmful effects for neuronal function. Specifically, calcium dysregulation during aging may provoke changes in cell excitability and synaptic plasticity, leading to functional lesions of the hippocampus. Current available evidence supports defective synaptic transmission as the cause of the progressive cognitive decline that accompanies advanced age, reflected by impaired LTP induction and higher susceptibility to LTD (124); LTD enhancement in advanced age may underlie the defects in rapidly acquired hippocampal-dependent memory that are an early characteristic of age-related cognitive decline. The activity-dependent sources of postsynaptic Ca^{2+} signals may shift during aging towards a decreased contribution of NMDA receptors and an increased role for RyR-mediated calcium release from intracellular stores and L-type calcium channels (124). In effect, whereas excitability decreases with age, the density of functional channels L-type calcium channels increases with age (389, 390). Interestingly, in aged animals RyR inhibition with ryanodine or emptying of ER calcium stores reduces the excitability decrease, allowing LTP induction even by low frequency synaptic activation (224), whereas inhibition of RyR or L-type channels inhibits LTD (225). These combined results suggest that neuronal RyR, which increase their activity as a consequence of redox modifications (209), presumably undergo significant stimulation by age-related oxidative stress, generating abnormal calcium signals that contribute to defective synaptic plasticity during aging (389). Furthermore, oxidative stress plus abnormally large postsynaptic calcium signals may jointly stimulate calcineurin activity, that increases during aging and further decreases NMDA receptor function via dephosphorylation. The age-related increase in calcineurin activity, which is reduced by L-type channel antagonists, has been attributed to enhanced calcium release from intracellular stores (196) or other factors, including the dephosphorylation of proteins involved in gene transcription, such as CREB, and activation of protein phosphatases (124).

Compared to other mammalian organs, the human brain and the heart are in particular danger of suffering oxidative stress with time due to their high oxygen consumption rates. The brain consumes 20% of the human body oxygen, yet it represents only 2% of the total body weight (152). Even more remarkably, the heart, an obligatory aerobic organ, consumes significantly more oxygen at a resting pulse rate than the brain and this consumption increases markedly during vigorous exercise (136). Accordingly, we will analyze next in further detail the conditions that promote cellular oxidative stress in these two organs and their harmful consequences for human health, evidenced by the emergence of worldwide life-threatening diseases.

C. Neurodegenerative diseases

The high oxygen consumption rates of the human brain necessarily imply substantial ROS generation. In particular, hydroxyl radical generation in neurons with high iron levels becomes especially dangerous to their function (167). The brain possesses low to moderate protective antioxidant systems, a characteristic that contributes to make neurons highly sensitive to oxidative stress, especially in conditions such as cerebral ischemia and reperfusion where the excessive ROS increase overwhelms endogenous antioxidant systems. For instance, a significant ROS increase occurs in pathological conditions such as stroke (265, 281); moreover, COX-2 inhibitors afford neuroprotective effects after cerebral ischemia (369), implying activation of COX-2-dependent ROS generation in this condition. In addition, oxidative stress may promote neurodegenerative diseases via perturbations of mitochondrial and ER function (399, 400).

As stated above, a considerable increase in ROS generation occurs during aging, which is the most significant risk factor for the development of neurodegenerative conditions. In fact,

excessive ROS generation contributes to the pathogenesis of several neurodegenerative diseases (185). Likewise, some neurodegenerative conditions such as Parkinson's disease seem to be associated with excessive NO production (104). As already pointed out, oxidative stress produces functional alterations of proteins involved in Ca^{2+} signaling and interferes with Ca^{2+} homeostasis. Consequently, during aging and in several neurodegenerative disorders, neurons have a decreased capacity to regain Ca^{2+} homeostasis after activity-dependent calcium signal generation (268). The resulting sustained $[Ca^{2+}]$i elevations are detrimental to nerve cells; in addition to their deleterious effects on mitochondrial and ER function, they activate calcium-dependent lipases and proteases that threaten cell life (298), or decrease neuronal excitability as described below. Additionally, ER alterations and ER stress seemingly contribute to the age-related dysfunction and the neuronal degeneration observed in some pathological conditions, including Alzheimer's disease (244). Moreover, as described above GSNO damages cerebellar granule cells by inducing sustained $[Ca^{2+}]$i elevations that arise in part from ER calcium depletion (158). Alzheimer's and Parkinson's disease are widespread age-associated neurodegenerative conditions that affect several million people worldwide. For this reason, we will focus here on how oxidative stress and abnormal calcium signaling may contribute to the generation and/or the progression of these two pathological conditions.

1. Alzheimer's disease. Alzheimer's patients exhibit progressive cognitive decline. Postmortem brain tissue has typical pathological features characterized by the presence of senile plaques composed of amyloid beta (Aβ) peptide aggregates and of intracellular neurofibrillary tangles composed primarily by hyperphosphorylated tau protein. Early oxidative damage and abnormal mitochondrial function have been associated with the pathogenesis of Alzheimer's disease. Tissue samples from Alzheimer's patients or aged brains show alterations in sphingolipid and cholesterol metabolism (83), with abnormal accumulation of long-chain ceramides and cholesterol. Noteworthy, early oxidative tissue damage occurs before the onset of significant brain plaque pathology and diminishes with disease progression (300), suggesting that compensatory changes occur during the progression of the disease to curb the cellular damage induced by oxidative stress. Thus, calcium homeostasis, synaptic plasticity, and memory may be affected in a manner analogous to normal aging and prior to the emergence of neurodegeneration. Persistent oxidative stress combined with the ensuing defective calcium signaling may alter synaptic transmission and induce cell death in Alzheimer's disease, causing the neuronal loss observed in different regions of the brain (227). Yet, it remains to be determined if defective calcium signaling is a causative agent or a consequence of Alzheimer's pathology.

Several reports indicate that Aβ is toxic to neuronal cells through the promotion of oxidative stress plus abnormal calcium homeostasis and signaling. Early studies done in brain tissue from Alzheimer's disease patients revealed increased calcium content associated with neurofibrillary tangles (291), Aβ-induced apoptosis due to abnormal calcium homeostasis (179), and membrane lipid peroxidation caused by enhanced production of HNE, which further disrupts calcium homeostasis (269). By enhancing CaN activity, Aβ decreases NMDA receptor function and impairs LTP (68). Yet the progression of Alzheimer's disease promotes the expression of CaN endogenous inhibitory proteins that are induced *in vitro* by oxidative stress and Aβ; CaN inhibition may promote tau phosphorylation, neurodegeneration, and tangle formation (79, 111). Studies in rat cortical neurons in culture have shown that Aβ enhances ER calcium release mediated by both IP_3R and RyR, suggesting a mechanistic link among Aβ accumulation, deregulation of ER calcium homeostasis, and apoptotic neuronal death in Alzheimer's disease (118). The occurrence of ER stress in Alzheimer's disease, however, may represent a possible neuroprotective mechanism rather than a pathway leading to neuronal deterioration (244). Other possible mechanisms for the ethiology of this disease have been proposed. These include disruption of calcium homeostasis by Aβ oligomer-induced calcium-conducting pores in neuronal membranes (204, 361), or neurotoxic effects due to formation of intermediate Aβ oligomers that accumulate in brain tissue of affected individuals (150). In fact, a specific Aβ oligomer impairs memory in transgenic mice independently of plaques or neuronal loss (237). Moreover, Aβ oligomers induce oxidative stress in hippocampal neuronal cultures, through a mechanism requiring calcium entry via NMDA receptors and that is effectively blocked by the Alzheimer drug memantine (89).

2. Parkinson's disease. Together with Alzheimer's disease, Parkinson's disease is the most prevalent neurodegenerative disorder of the elderly. The clinical characteristics of Parkinson's disease are progressive rigidity, resting tremors, slowness of voluntary movement, and postural instability. The neuropathological hallmarks of Parkinson disease are progressive loss of the nigrostriatal dopamine-containing neurons, which have their cell bodies in the *substantia nigra pars compacta* and their nerve terminals in the *striatum*, plus the presence of Lewy bodies, iron-containing cytoplasmic aggregates that immunostain for alpha synuclein and ubiquitin.

Increased $[Ca^{2+}]$i and oxidative stress appear to contribute to the neurodegeneration observed in Parkinson's disease (80, 88, 185, 359, 360). Cellular calcium overload may cause the death of vulnerable neurons in patients with Parkinson's disease or other neurodegenerative conditions, while NOX-generated ROS are critical for its induction in animal models of Parkinson's disease (131). Brain tissues from patients with Parkinson's disease display decreased GSH levels and increased protein oxidation in human *sustantia nigra pars compacta* relative to other brain regions, with oxidative damage to DNA, lipids, and proteins (123, 309). Moreover, diminished intracellular GSH levels correlate with dopaminergic cell loss in Parkinson's disease, suggesting that dopaminergic neurons are especially sensitive to oxidative stress (152). Significantly, transfection of PC12 cells with antisense oligomers directed against the rate-limiting enzyme in GSH synthesis decreases GSH levels and prompts an increase in ROS and $[Ca^{2+}]$i (193). These findings suggest that decreased GSH levels in Parkinson's disease may enhance cell death through the disruption of calcium homeostasis caused by oxidative stress. Furthermore, the large calcium signals elicited by calcium influx through NMDA receptors during glutamate-induced excitotoxicity may produce abnormally high NO production, which may also con-

tribute to induce dopaminergic neuronal death in Parkinson's disease (20).

Recent studies indicate the ER unfolded protein response is activated in Parkinson's disease: this activation closely associates with alpha-synuclein accumulation and aggregation (175). Furthermore, results obtained in brains manifesting sporadic Parkinson's or Alzheimer's disease indicate that PDI is *S*-nitrosylated. This redox modification inhibits PDI activity, leading to the accumulation of polyubiquitinated proteins and activation of the unfolded protein response, and suppresses the PDI-mediated attenuation of neuronal cell death caused by ER stress, misfolded proteins or proteasome inhibition (400).

The generation of dopamine oxidation products, which are major components of neuromelanin (the dark pigment that accumulates with age in substantia nigra), is a particularly relevant feature of Parkinson's disease. It has been proposed that dopamine oxidation products generated by COX-1 (constitutively present in microglia), and particularly by COX-2, which is present in dopaminergic neurons of the substantia nigra of Parkinson's disease patients, have a key role in the pathogenesis of this disease (387). Defective calcium homeostasis and signaling may induce mitochondrial calcium overload, a condition that by enhancing mitochondrial ROS production promotes the generation of dopamine oxidation products. Moreover, some inhibitors of the mitochondrial respiratory chain promote Parkinson's disease-like symptoms and signs of neuronal oxidative stress; they do so presumably via generation of dopamine oxidation products that promote H_2O_2 release at Complex I in brain mitochondria (449). Noteworthy, mitochondrial Complex I activity is frequently defective in individuals affected with Parkinson's disease (364).

Postmorten analysis of brain tissue from Parkinson's disease patients has revealed increased iron content in *substantia nigra* and individual dopaminergic neurons (279, 301). An increased iron content might produce a decrease of intracellular GSH levels, with the ensuing intensification of oxidative stress and generation of potentially damaging free radicals, and might also promote Lewy body formation (201, 202). Moreover, chronic ferritin elevation in murine midbrain dopamine-containing neurons causes their progressive age-related neurodegeneration (203).

The surviving dopaminergic neurons in Parkinson's disease presumably possess high levels of calbindin, a redox-sensing calcium binding protein that ameliorates the neuronal cell death caused by excessive $[Ca^{2+}]i$ when overexpressed in neurons or after *in vivo* infection of animals with calbindin-expressing viral vectors (317). These findings indicate that strategies that limit the emergence of abnormally elevated calcium signals may be useful in the treatment of Parkinson's disease patients, as discussed below.

3. Therapeutic approaches. Sustained oxidative stress, with the ensuing alterations in Ca^{2+} signaling and homeostasis, contributes to the pathogenesis of several neurological disorders, such as Alzheimer's and Parkinson's diseases. Accordingly, among the different therapeutic strategies that are currently investigated, decreasing oxidative stress and restoring neuronal Ca^{2+} homeostasis and Ca^{2+} signaling may retard in principle, or even prevent the neurodegeneration that occurs in these neurological disorders. For example, antioxidants and dietary supplementation with omega-3 fatty acids have beneficial effects in neurodegenerative diseases, among other pathologies (270). Therapies employing antioxidant agents may also delay age-related neuronal dysfunction and the pathological processes that elicit Alzheimer's disease (250). Noteworthy, dietary cysteine supplementation may have potential therapeutic effects on brain function decay during aging. In fact, clinical trials indicate that cysteine supplementation improves parameters that degenerate with age, such as skeletal muscle functions among others, suggesting that a deficit in cysteine due to suboptimal dietary consumption may have deleterious effects during aging and in some pathological conditions (99). Likewise, strategies that afford protection against the excessive $[Ca^{2+}]i$ elicited by decreased GSH levels and oxidative stress or excessive calcium influx may be effective for neuronal survival. As an example, memantine (49), a moderate-affinity, uncompetitive NMDA receptor antagonist, has been shown to slow the rate of decline in Alzheimer's disease patients. Interestingly, it has been proposed that the decreased cell excitability and synaptic transmission that occurs in aged neurons may represent attempts to prevent $[Ca^{2+}]i$ from reaching levels that can set off neurodegenerative diseases (268). Yet, this survival strategy entails significant reduction in normal neuronal functions that limit its eventual beneficial effects. To combat Alzheimer's disease, many drugs are currently being tested in clinical trials, including drugs that inhibit Aβ aggregation, antioxidants, and calcium channel blockers, among others (337). On the other hand, results obtained in cellular model systems indicate that blockage of Aβ-induced ion channels at the cell surface effectively prevents cell death (11). This potential therapeutic strategy has not been tested in animal models of Alzheimer's disease, so its potential applicability to human patients is uncertain.

A recent report indicates that PGC-1α null mice are especially sensitive to the neurodegenerative effects of agents that induce oxidative stress in the *substantia nigra* or the hippocampus, whereas increasing PGC-1α levels protects cultured neuronal cells incubated with hydrogen peroxide (368). Accordingly, increasing PGC-1α levels in the brain (not a simple task) may help to counter the cellular oxidative damage observed in Parkinson's and Alzheimer's diseases. Yet, as pointed out elsewhere (368), PGC-1α is highly inducible via common pathways that engage calcium and cyclic AMP signaling, a property that opens possibilities to develop PGC-1α-inducing drugs in the brain. New therapies for neurological diseases prevalent among the elderly and that presently lack effective treatments (171, 363) may arise from this approach.

D. Cardiovascular pathologies

The characteristically high oxygen consumption displayed by the heart, which increases markedly during vigorous exercise, may cause cellular oxidative stress if antioxidant defense systems fail to balance ROS generation properly. In fact, oxidative stress in the heart is a hallmark of pathological conditions, such as ischemic heart disease and cardiac hypertrophy. The main sources of ROS generated in cardiac tissue under these pathological conditions include NOX, XO, uncoupled NOS, and mitochondria-generated ROS/RNS.

1. Ischemia–reperfusion injury. In the heart, a few minutes of ischemia can increase up to 10 μM the time-averaged intracellular [Ca^{2+}] (452). Activation of Ca^{2+}-dependent hydrolytic enzymes including proteases and phospholipases follows, causing irreversible degradation of essential cellular components. With the onset of reperfusion, [Ca^{2+}] can increase even further and promote a mitochondrial [Ca^{2+}] rise, that by stimulating the opening of the mitochondrial permeability transition pore can eventually lead to cardiac cell death (91). In parallel to the [Ca^{2+}] increase, a few minutes of ischemia also induce a ROS increase in cardiac myocytes, while an additional ROS burst occurs at the time of reperfusion (21, 453). By modifying calcium transport systems, as described in Section IV, this ROS increase may further contribute to increase sequentially cytoplasmic and mitochondrial [Ca^{2+}], leading to enhanced mitochondrial ROS generation. Consequently, Ca^{2+} and ROS would influence each other, leading to mutual regenerative increases in their concentrations in a complex interrelation that ends by killing the cell (45).

Cardiac preconditioning constitutes a powerful means of protecting the myocardium against ischemia–reperfusion damage. Short episodes of ischemia (292), rapid pacing (95), exercise (94), angiotensin II (86), volatile anesthetics (383), or other pharmacological agents confer resistance against the heart damage produced by a subsequent ischemic episode. Irrespective of the initial maneuver used to induce preconditioning, ROS production seems to be crucial to the genesis of protection (69, 405); furthermore, ROS by themselves can effectively induce preconditioning in the absence of ischemia or other stimuli (398). Increasing extracellular [Ca^{2+}] also results in preconditioning (299, 328). As discussed in the case of ischemic reperfusion injury, ROS and Ca^{2+} may act synergistically to induce preconditioning.

Several studies support a role for NADPH oxidase as a source of ROS during preconditioning. Thus, hearts from NOX knockout animals are less susceptible to early ischemic preconditioning than controls (23), whereas apocynin, a NOX inhibitor, abolishes angiotensin II-induced preconditioning both in conscious rats (214) and isolated hearts (87). Apocynin is a well-known NOX inhibitor; therefore, these results suggest that this enzyme has an important role in cardiac preconditioning. Although the molecular targets of NOX-derived ROS remain unidentified, recent results suggest that RyR2 Ca^{2+} release channels may be one of the links connecting ROS and Ca^{2+} in their synergistic promotion of cardiac preconditioning. Preconditioning tachycardia increases NOX activity, which by promoting RyR2 *S*-glutathionylation enhances RyR2 activity (345). Yet the precise mechanisms linking RyR2 *S*-glutathionylation with the increased calcium release activity remain unknown. Among other possibilities, redox enhancement of RyR2 activity might be caused by changes in the binding of calmodulin (17), which inhibits the channel, or of FKBP12.6 (447), which stabilizes the channel, since binding of both molecules is redox modulated.

2. Cardiac hypertrophy and heart failure. Myocardial hypertrophy, which starts as an adaptive mechanism to hemodynamic stress, encompasses structural and functional changes in the heart. If the stress is permanent, the initial adaptive process progresses to chronic heart failure. As detailed below, both Ca^{2+} and ROS are important inducers of cardiac hypertrophy (16, 382).

Several calcium channels and transporters undergo activity changes during hypertrophy (277, 287, 407); yet, their roles in the development of hypertrophy are not well established. Most models of cardiac hypertrophy report a decrease in both protein content and activity of SERCA2a (375), while opposite findings—an increase in protein content and activity—have been reported for the NCX (276, 375). In contrast, studies to analyze the participation of L-type calcium channels in the development of cardiac hypertrophy and heart failure have yielded mostly negative results (287). Several reports have demonstrated that calcineurin, a Ca^{2+}-dependent phosphatase that promotes the dephosphorylation and nuclear translocation of the transcription factor NFAT, has a role in mediating cardiac hypertrophy and progressive heart failure (421). Whereas a connection among L-type current, calcineurin activity levels and cardiac remodeling has not been described, sustained cardiac SR depletion during hypertrophy stimulates Ca^{2+} entry through TRP channels, which by enhancing calcineurin activity (52, 293) stimulates the translocation of NFAT to the nucleus, where it activates the transcription of genes that promote cardiomyocyte hypertrophy (421). Other Ca^{2+}-dependent kinases, such as the delta C isoform of CaMKII (444) and PKC (96) are also activated in cardiac hypertrophy and induce dilated cardiomyopathy and heart failure. The signaling pathways activated by these kinases are under active investigation; a detailed analysis of this specific subject is beyond the scope of this review. Activation by ROS of other signaling kinases and transcription factors also causes hypertrophy and heart failure (305, 382). Importantly, in addition to its alleged protective role during preconditioning, the activity of NOX enzymes may be crucial for the development of cardiac hypertrophy. Cardiomyocytes from patients with myocardial infarction or heart failure display increased NOX activity (24, 53, 129, 241). Different agents that activate NOX, such as angiotensin II, noradrenaline, and mechanical overload (65, 290) promote cardiac hypertrophy (24, 241, 430). Additionally, the human failing heart displays increased NOX activity, as recently reported (259).

In end-stage human and canine heart failure, XO expression and activity increases in cardiac muscle (57, 109, 401). Noteworthy, XO inhibition reduces oxidative stress and improves both vascular and myocardial function in experimentally induced heart failure, and also reverses cardiac remodeling after ischemic injury (446).

3. Diabetic cardiomyopathy. Clinically, diabetes mellitus is a major risk factor for multiple myocardial infarctions and death from myocardial infarction (199). The diabetic condition is associated to a more oxidized cellular environment that in the myocardium results in a shift in the redox state of cellular proteins. Hyperglycemia-induced ROS generation is due in part to excessive pyruvate generation, which increases ROS generation in the mitochondria (189). Several ROS-generating enzymes, however, including NOS, NOX, and XO, also display increased activity in diabetic subjects. Moreover, in isolated cardiac myocytes, early glycated proteins stimulate ROS generation via PKC-dependent NOX activation (442). These authors showed that NOX activation increases ROS generation, which promotes the translocation to the nucleus of NF-κB, a

redox sensitive transcription factor, and promotes a significant increase in the mRNA content of atrial natriuretic factor, a hypertrophic marker.

The reported protective effects of XO inhibition indicate that decreasing oxidative stress ameliorates heart failure. Likewise, PGC-1α may exert protective effects in the heart, as noted above for neurodegenerative diseases. In fact, PGC-1α null mice exhibit cardiac dysfunction during aging (10). It remains to be determined, however, if oxidative stress reduction normalizes Ca^{2+} signaling in heart failure, and what are the detailed molecular basis that underlie the interaction between ROS and Ca^{2+} in cardiac pathologies. In addition, several reports have described a reduction in SERCA expression and activity during aging and in a variety of pathophysiological conditions, including heart failure; noteworthy, SERCA gene transfer has proven effective in restoring contractile function in failing heart muscle (311). These studies highlight the potential use of SERCA gene transfer as a therapeutic strategy in heart failure.

In summary, these combined studies point to abnormal Ca^{2+} signaling and oxidative stress as inducers of cardiac pathologies. Yet, in spite of the increasing evidence for a role of oxidative stress as a causative agent in several cardiovascular diseases, it has not been possible to establish well-defined antioxidant therapies (354, 382). This dearth of effective antioxidant therapies is not surprising, given the narrow limit between physiological cardiac redox signaling and oxidative stress. Moreover, the highly ordered spatial distribution and interactions of the cellular components makes difficult for nonspecific and wide-spectrum antioxidant molecules to reach only a few desired targets among all the multiple possible targets present in cardiac cells. Undoubtedly, further research is required to decipher the fine-tuning mechanisms that control cardiac redox pathways, in order to direct antioxidant therapies to the desired molecules without affecting other possible molecular substrates.

VI. CONCLUDING REMARKS

The studies reviewed here, most of them published in the last decade, demonstrate that calcium signals in conjunction with ROS/RNS influence a variety of physiological signaling pathways that are essential for cellular function. Thus, as illustrated in Fig. 15, ROS/RNS modify the functional properties of calcium-dependent enzymes and transcription factors and elicit calcium signals through redox modifications and subsequent stimulation of ER calcium release channels. The resulting calcium signals can exert positive feedback on ROS/RNS production and calcium signal generation, via CICR, and may promote gene expression among other cellular responses (Fig. 15), as discussed in the text. Moreover, excess ROS/RNS generation promotes cellular oxidative stress, and evidence is emerging indicating that this non-physiological cellular state produces abnormal calcium signals, which prompt diseases states or can cause cell death. The number of joint cellular targets of calcium and ROS/RNS keeps increasing. Consequently, significant knowledge regarding the specific protein sites that undergo reversible or irreversible calcium-induced redox modifications, and how cells regulate or fail to regulate them, is likely to emerge in the coming years. In particular, understanding the detailed molecular mechanisms whereby oxidative stress conditions prompt the generation of abnormal calcium signals is bound to generate new therapeutic approaches for many current human diseases.

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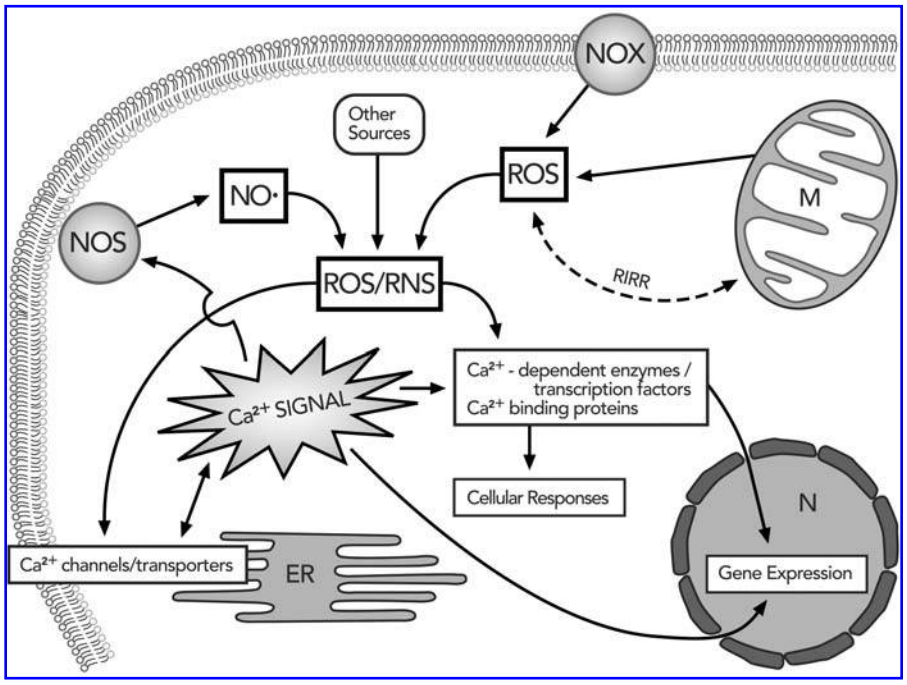

FIG. 15. Cellular sources and crosstalk between calcium and ROS/RNS signaling pathways. ROS/RNS elicit calcium signals via redox modifications of the ER calcium release channels, and modify the functional properties of calcium-dependent enzymes and transcription factors. The resulting calcium signals can exert positive feeback on ROS/RNS production and calcium signal generation, and promote gene expression among other cellular responses.

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ABBREVIATIONS

Aβ peptide, amyloid beta peptide; AC, adenylyl cyclase; BH_4, tetrahydrobiopterine; BKCa, high conductance calcium activated potassium channel; $[Ca^{2+}]i$, intracellular calcium concentration; cADPR, cyclic ADP-ribose; CaM; calmodulin; cAPK, cyclic AMP-dependent protein kinase; CICR, calcium-induced calcium-release; COX, cyclooxygenase; Cys-less, cysteine residue lacking protein; DTT, dithiothreitol; eNOS, endothelial nitric oxyde synthase; ER, endoplasmic reticulum; FKBP12, FK506 binding protein; GPX, glutathione peroxidase; GSSG, glutathione disulfide: GSH, glutathione; GSNO, *S*-nitrosoglutathione; HNE, 4-hydroxy-2,3-nonenal; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxyde synthase; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptors; LOX, lipoxygenase; NCX, sodium/calcium exchanger; NCKX, sodium/calcium/potasium exchanger; NMDA, N-methyl D-aspartate; nNOS, neuronal nitric oxyde synthase; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidases; PDI, protein-disulfide isomerase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SH, sulfhydryl; SOCE, store-operated calcium entry; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; TRP, transient receptor potential; XO, xanthine oxidase.

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