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REVIEW

Cross-Talk between Reactive Oxygen Species and Calcium in Living Cells

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Abstract—The results of many investigations have shown that calcium is essential for production of reactive oxygen species (ROS). Elevation of intracellular calcium level is responsible for activation of ROS-generating enzymes and formation of free radicals by the mitochondria respiratory chain. On the other hand, an increase in intracellular calcium concentration may be stimulated by ROS. H_2O_2 has been recently shown to accelerate the overall channel opening process in voltage-dependent calcium channels in plant and animal cells. The 1,4,5-inositol-triphosphate-receptors as well as the ryanodine receptors of sarcoplasmic reticulum have also been demonstrated to be redox-regulated. Activity of Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers of animal cells are modulated by the intracellular redox state. Simultaneously, Ca^{2+} may activate antioxidant enzymes, such as plant catalase and glutathione reductase, and increase the level of superoxide dismutase in animal cells. Reviewed data support the speculation that Ca^{2+} and ROS are two cross-talking messengers in various cellular processes.

Key words: antioxidant enzymes, calcium, calcium channels, ROS, ROS-generating enzymes, second messenger

Reactive oxygen species (ROS) often are considered as tissue-damaging agents, especially in the presence of elevated cytosolic Ca^{2+} ($[Ca^{2+}]_{in}$). However, cells synthesize prooxidants such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) during normal activities. Like other posttranslational modifications, oxidation of amino acid residues in proteins promoted by ROS alters properties of a number of cellular proteins involved in signal transduction, such as protein kinases, protein phosphatases, and transcription factors. Redox-dependent regulation of components of the intracellular Ca^{2+} homeostasis may influence the direction and/or the efficiency of Ca^{2+}-signaling pathways [1-6]. On the other hand, a number of ROS-generating and antioxidant systems of living cells are calcium-dependent [1-6]. So, it can be though that calcium level oscillations and ROS formation/deactivation processes are intimately linked with each other. It could be speculated that Ca^{2+} and ROS are two cross-talking messengers in various cellular processes. Here we review experimental data supporting this speculation.

Abbreviations: BAPTA) 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BAPTA AM) acetoxymethyl ether of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; EGTA) ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; ROS) reactive oxygen species.

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REASONS FOR THE EXISTENCE OF Ca^{2+}/ROS INTERACTION

Polychaete worms of the family *Polynoidae* provide an example of a ROS-dependent bioluminescent system. Their bioluminescence is impulse-like and is stimulated by exogenous, presumably mechanical, stimuli. Special epithelial cells—photocytes—produce the light. The photoprotein, which is called polynoidin, is situated in the photosomes, which are made of tubules of endoplasmic reticulum. The photoprotein produces light upon reaction with $O_2^{\cdot-}$ [7]. Every action potential of a photocyte's plasmalemma leading to $[Ca^{2+}]_{in}$ elevation is accompanied by a light flash enduring near 100 msec. In the course of the action potential, $O_2^{\cdot-}$ is generated by a photosomal enzyme [8]. During repetitive stimulation of bioluminescent flashes their amplitude begins with elevation and slowly delayed like muscle contraction amplitude during dentate tetanus. Is this a specific phenomenon? There are a number of other data suggesting that every action potential is accompanied by $O_2^{\cdot-}$ generation not only in polynoid annelids. Thus, ultra weak chemiluminescence of contracting frog heart is visibly intensified in systolic phases of its rhythmic contraction [9]. Isolated frog muscles obtained from various body locations radiate at low intensity when stimulated [9]. Pulsed electric excitation of frog sciatic nerve caused photon emission [9]. It is well

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known that in all these processes plasmalemmal depolarization leads to the $[Ca^{2+}]_{in}$ elevation, and low intensity light emission of living organisms is mostly attributable to the oxidative processes accompanied by ROS formation [6, 9].

Chelation by BAPTA suppressed H_2O_2 formation in the human keratinocyte cell line HaCaT, and the kinetics of the rise and decay of $[Ca^{2+}]_{in}$ were similar to those of H_2O_2 [10]. H_2O_2 generation by these cells intensified during elevation of $[Ca^{2+}]_{in}$ and extracellular calcium concentration ($[Ca^{2+}]_{out}$) [11]. ROS production by marine invertebrates (*Sycon* sponges and *Aiptasia* sea anemones) depends on $[Ca^{2+}]_{in}$ and $[Ca^{2+}]_{out}$, too [12].

On the other hand, the increase in $[Ca^{2+}]_{in}$ might be stimulated by ROS. It has been recently demonstrated that H_2O_2 caused a dose-dependent significant rise in $[Ca^{2+}]_{in}$ of human and rat endothelial cells [13], of peripheral blood mononuclear cells [14], and of *Arabidopsis* higher plant guard cells [15]. $[Ca^{2+}]_{in}$ elevation in cultured cortical neurons was blocked by antioxidants α-tocopherol and U83836E [16]. Cortical neurons from young rats (9-30-day-old) showed a long-lasting, sustained elevation of basal $[Ca^{2+}]_{in}$ after a single, paired application of depolarization and oxidation with either H_2O_2 or O_2. Combined application of depolarization and oxidation by H_2O_2 (0.03%) to undifferentiated rat pheochromocytoma (PC12) neurosecretory cells markedly potentiated the subsequent Ca^{2+} signals in response to K^+ depolarization [4]. Therefore, an intimate cross linkage between Ca^{2+} and ROS may exist in living cells.

CALCIUM AND ROS-GENERATING ENZYMES

There are many intracellular sources of ROS. They include the electron transport chain of mitochondria and a wide array of extramitochondrial enzymes. These include cell-surface NADPH-oxidase [1-5], myeloperoxidase [17], NO-synthase [2], cyclooxygenase, lipoxygenases, xanthine oxidase, monoamine oxidases, tyrosine hydroxylase, and L-amino-acid oxidase [1-5]. Another cellular ROS-generating site is the endoplasmic reticulum, where $O_2^{\cdot-}$ is generated by a leakage of electrons from NADPH-cytochrome-P450 reductase [3]. Activation of neutrophil oxidases, including NADPH-oxidase, is $[Ca^{2+}]_{in}$-dependent [18]. Preincubation of human neutrophils with chelators of intra- or extracellular Ca^{2+} inhibited respiratory burst activity and decreased the generation of toxic oxygen metabolites [19]. Influx of Ca^{2+} through voltage-gated channels activates the NADPH-oxidase in murine microglial cells during reoxygenation [20]. Cell-surface NADPH-oxidase of non-stimulated animal cells consists of a membrane-spanning, heterodimeric cytochrome *b* consisting of a large β-subunit (gp91-phox) and a smaller α-subunit (p22-phox) associated with two proteins located in the cytosolic fraction of non-stimulated cells—p47-phox and p67-phox [21]. It activates by calcium-dependent proteins—protein kinase C (PKC) and phospholipase A_2 (PLA_2) [3, 22-24]. Diacylglycerol (DAG) formed during hydrolysis of phospholipids activates PKC, which phosphorylates p47-phox and p67-phox. Assembling of active NADPH-oxidase complex results from association of phosphorylated cytosolic subunits with membrane-spanning cytochrome *b* [25, 26]. Free arachidonate formed during phospholipids hydrolysis is the immediate activator of NADPH-oxidase [21].

NADPH-oxidase and its homologs are widely expressed among living organisms. They present in practically all cell types of animal [27] and plant [28] tissues. Furthermore, mesocaryotic organisms, such as *Chattonella marina*, one of the most toxic red tide phytoplankton, have a homolog of NADPH-oxidase, namely, gp91-phox subunit [29]. It is logical that this subunit is responsible for ROS excretion by these algae. Plant homologs of the gp91-phox NADPH-oxidase, identified in tomato (*Lycopersicum esculentum* Mill.) and tobacco (*Nicotiana tabacum* var. *Samsun*) plasma membranes, can produce $O_2^{\cdot-}$ in the absence of additional cytosolic components and are stimulated directly by Ca^{2+} [30].

A number of data [23, 24] have shown that activities of other ROS-generating enzymes are regulated by $[Ca^{2+}]_{in}$ directly or indirectly, too. Myeloperoxidase catalyzing hypochlorite ion ($HOCl^-$) formation from Cl^- and H_2O_2 [17] contains a calcium-binding site [31] that is essential for its activity [32].

CALCIUM AND ROS GENERATION IN MITOCHONDRIA

Ca^{2+} uptake into mitochondria is a necessary step for ROS formation [33]. In *in vitro* experiments, it has been found that with an excess of Ca^{2+} in the medium, isolated mitochondria can generate ROS from the respiratory chain [34]. After exposure of neural cells to 2 mM 3-nitropropionic acid (3-NP, an irreversible inhibitor of succinate dehydrogenase, which increases H_2O_2 and $ONOO^-$ production by mitochondria), the $[Ca^{2+}]_{in}$ rose rapidly and progressively. The intracellular Ca^{2+} chelator BAPTA AM largely prevented apoptosis induced by 3-NP. Similarly, nifedipine (a blocker of L-type voltage-dependent calcium channels) and dantrolene (a blocker of calcium release from endoplasmic reticulum stores) significantly attenuated 3-NP-induced apoptosis. So, $[Ca^{2+}]_{in}$-decreasing agents prevent H_2O_2 and $ONOO^-$ generation by mitochondria [35]. Ca^{2+} stimulated H_2O_2 formation by diaphragm mitochondria, and inhibitors of mitochondrial PLA_2 blocked the enhanced H_2O_2 generation. Arachidonic acid (the principal metabolic product of phospholipid hydrolysis by PLA_2) increased mitochon-

drial H_2O_2 formation by interacting with complex I of the electron transfer chain [36]. On the other hand, oxidation of thiols in mitochondrial membrane proteins induce Ca^{2+} release from mitochondria [37]. Staurosporine inducing mitochondrial ROS ($ONOO^-$ in particular) formation, induced an early increase in $[Ca^{2+}]_{in}$ followed by delayed increase of mitochondrial Ca^{2+} level [38]. Prooxidants and menadione, inducers of mitochondrial $O_2^{\cdot-}$ generation, cause a rapid Ca^{2+}-elevation in thymocytes and T cell hybridoma cells [39]. In isolated mitochondria, ROS and intramitochondrial Ca^{2+} can act together to trigger the opening of the mitochondrial permeability transition pore (mPTP) [40]. Mitochondrial membrane permeability transition induced by inorganic phosphate, uncouplers, or prooxidants such us *tert*-butyl hydroperoxide and diamide is caused by a Ca^{2+}-stimulated production of ROS by the respiratory chain [41]. Studies with submitochondrial particles have demonstrated that the binding of Ca^{2+} to these particles induces lipid lateral phase separation, leading to disorganization of respiratory chain components, favoring ROS production and consequent protein and lipid oxidation. The ROS attack to membrane protein thiols produces a cross-linkage reaction, which may open membrane pores upon Ca^{2+} binding [41].

ROS AND CALCIUM TRANSPORT

It has been shown that H_2O_2, $O_2^{\cdot-}$, and singlet oxygen takes parts in the activation of plasmalemmal calcium channels in animal [42] and plant cells [43] as well as 1,4,5-inositol-triphosphate- and ryanodine-sensitive calcium channels of sarcoplasmic reticulum [42]. Simultaneously, $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ presumably sometimes (but not always) inhibit Ca^{2+}-ATPases of sarcoplasmic reticulum and Na^+/Ca^{2+} exchangers [42]. The influence of various ROS on Ca^{2+}-transport systems is different. In particular, it has been found that $HOCl^-$ induced inhibition whereas H_2O_2 induced stimulation of the Na^+/Ca^{2+} exchanger [42]. H_2O_2 resulting from Ca^{2+}-dependent activation of plant NADPH-oxidase leads to $[Ca^{2+}]_{in}$ elevation and activates plasmalemmal calcium channels [43].

CALCIUM AND ANTIOXIDANT ENZYMES

Calmodulin, a ubiquitous calcium-binding protein, binds to and activates some plant catalases in the presence of Ca^{2+} [44]. These results document that Ca^{2+}/calmodulin can downregulate H_2O_2 levels in plants by stimulating the catalytic activity of plant catalases. Thus, Ca^{2+} has a dual function in regulating ROS homeostasis [44]. Ca^{2+} may be involved in plant tolerance to heat stress [45]. Plants treated with exogenous Ca^{2+} under heat stress had higher catalase and glutathione reductase activities than untreated plants. Lesser amounts of malondialdehyde, a product of lipid peroxidation, accumulated in Ca^{2+}-treated plants than in untreated plants during extended periods of heat stress. The results suggest that exogenous Ca^{2+} treatment enhanced heat tolerance due to maintenance of antioxidant activities and a decrease in membrane lipid peroxidation [45]. The calcium ionophore ionomycin increased the level of superoxide dismutase in cultured cortical neurons from embryonic rats [46]. In rat cerebellar neurons it increased oxidative metabolism and decreased the content of non-protein thiols [47]. When K^+-depolarization and histamine—agents increasing $[Ca^{2+}]_{in}$—were applied to PC12 cells in combination with 0.03% H_2O_2, these stimuli significantly reduced the increase in ROS [4]. To explain these data we suppose the existence of special Ca^{2+}-triggered pool of low molecular antioxidants protecting neurons from ROS following $[Ca^{2+}]_{in}$ elevation. Thus, it is possible that ROS may take parts in supporting calcium homeostasis and in regulation of a number of calcium-dependent physiological processes.

The reviewed data suggest that cross-talk between Ca^{2+} and ROS exists not only in pathological processes but also in normal cell functioning. It is present in organisms of various phylogenetic levels—from lower multicellular invertebrates to higher plants and animals including humans. Taking into account these data, it may be interesting to make clear the role of calcium-dependent ROS formation in regulation of $[Ca^{2+}]_{in}$ kinetics during the impulse electrogenesis in skeletal and heart muscles and synaptic transmission.

But is the cross-talk between these two messengers universal? Whether it is present in bacteria, fungi, algae, and unicellular animals or not is still unknown. We think that careful investigation of Ca^{2+}/ROS homeostasis of these organisms should be done.

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