The biology of oxygen and calcium

The biological importance of molecular oxygen and calcium must make them two of the most interesting elements active in living organisms. Both have unique physical properties that underpin their biological function [1,2]. Calcium and oxygen share one fundamental biological property: they are both toxic.

Oxygen acts as the final electron donor in many biological oxidation pathways, including respiration, receiving four electrons in a stepwise manner via partially reduced intermediates. It is these partially reduced intermediates (superoxide, hydrogen peroxide and the hydroxyl radical), together with photoenergized molecular oxygen (singlet oxygen), that are responsible for the toxicity of oxygen [1]. These moieties, which are collectively known as activated oxygen species, are highly reactive oxidizing agents and contribute to the oxidative destruction of macromolecules [1]. In plants, in which the chemistry and biochemistry of photosynthesis potentiates activated oxygen production, protection against these species plays a crucial role in the resistance to environmental stress. Many conditions that limit plant productivity do so via enhanced oxygen activation either directly (e.g. ozone, herbicide treatment, UV radiation, transition metal toxicity) or indirectly (drought, chilling, herbicide treatment, anoxia, senescence) [3–5].

Considerable effort has been expended in research aimed at understanding the mechanism and consequence (economic and ecological) of oxidative stress in plants [3–6]. Characterization of molecular responses has shown that regulation of the complex protective mechanism that defends against oxygen activation is sensitive to many stress conditions. However, the process by which plant cells perceive oxidative stress has not been elucidated.

Whereas the toxicity of oxygen is largely a consequence of its chemistry, the toxicity of calcium is mainly the result of its biological role as a secondary messenger in animals [2] and plants [7–10]. The signal transduction system by which many chemical and environmental stimuli are perceived (and thence acted upon) commonly involves elevations in cytosolic calcium concentration. Whether this calcium elevation is transient or sustained, it is understood to result in modulation of enzyme activity and gene expression and is absolutely required for these observed responses. For cytosolic calcium to act as a secondary messenger, cells maintain low cytosolic calcium concentrations, typically 100 nM. This is achieved by transport of calcium from the cell and sequestration of calcium in organelles. Very large concentration gradients (3–6 orders of magnitude) therefore exist across the plasma and organelle membranes. This poise allows fine alterations in cytosolic calcium concentration to be translated into molecular responses. However, conditions that disrupt the cells' ability to maintain these concentration gradients cause uncontrolled cytosolic calcium elevation and uncontrolled modulation of cellular processes, including activation of catabolic enzymes [11,12]. The role of calcium as a secondary messenger inevitably means that uncontrolled perturbation of calcium transport within cells damages metabolism and is potentially lethal [2,11–13].

The connection between oxygen and calcium

In the past, the biology of oxygen and calcium were considered largely independently. During the last 15 years, however, evidence has emerged that there is a crucial link, if not an intimate relationship, between oxidative stress and calcium homeostasis.

In the early 1980s, work largely conducted at the Karolinska Institute, Sweden, demonstrated in hepatocytes that agents that promote oxidative stress also cause an increase in cytosolic calcium concentrations [14,15]. Owing to the temporal nature of the coupling between stress and calcium elevation, it was concluded that elevated cytosolic calcium is a primary result of oxidative challenge and not an artifact of gross cellular dysfunction [11–16]. It was also established that cytosolic calcium homeostasis is sensitive to the oxidation status of the glutathione pool [17] via the plasma membrane Ca\(^{2+}\)-ATPase [18].

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Summary, it was concluded that increased activation of oxygen species results in an increase in the ratio of oxidized (GSSG) to reduced (GSH) glutathione. This causes a reduction in the activity of calcium transport proteins of cell membranes and a resultant rise in cytosolic calcium concentration, which has a profound effect on enzyme activity, particularly degradative enzymes (proteases, phospholipases and endonucleases) [19], leading to calcium-dependent cell death.

In the past 5 years there has been a very large number of reports (hundreds) characterizing the coupling of oxidative stress agents in animal cells with altered calcium homeostasis. For example, hydrogen peroxide has been shown to cause elevations in cytosolic calcium in human endothelial cells [20–22], rat phaeochromocytoma cells [23], and monkey renal tissue [24]. Ozone has been shown to have similar effects on tracheal epithelium [25], and calcium influx is implicated in ozone-induced bronchial constriction [26]. Superoxide [21], cumene hydroperoxide [20] and tert-butyl hydroperoxide [13,27] have also been shown to stimulate cytosolic calcium concentrations in animal tissues.

There is contradictory evidence for the cause of calcium disruption. A link with thiol oxidation status is implicated in some publications [20,28], but evidence that the hydroxyl radical [21] or lipid hydroperoxides [22] are involved is also reported. Evidence also exists that Ca²⁺-ATPases are directly inhibited by the superoxide radical [29,30].

Despite this confusion, it is now abundantly clear that modulation of calcium homeostasis is a ubiquitous event early in the biology of oxidative stress. It is also clear that the pathology of oxygen is critically linked to the pathology of calcium. In addition, it is becoming clear that calcium perturbation by oxidative challenge is a mechanism by which cells are able to sense and respond to enhanced oxygen activation [12,31].

Plants

Photosynthesis and the sessile nature of plants predispose them to the harmful effects of both oxygen and calcium. The photosynthesis process causes oxygen evolution and the formation of oxygen radicals [32]. Environmental conditions that cause increased activation of oxygen, such as drought, heavy metal contamination, increased UV-B radiation, anoxia or atmospheric air pollution, must be contended with if a plant is to survive.

Likewise, the calcium that arrives at a leaf in the transpiration stream must be safely sequestered, as calcium is not translocated out of a leaf once it has arrived [33]. This appears to be achieved by transport into the vacuole of epidermal cells, which can accumulate to the order of 150 mM free calcium [34].

In comparison with animal cells, plant cells experience far higher cellular concentrations of oxygen (250 μM versus 0.1 μM for animals) [3], and have calcium gradients across the tonoplast probably two orders of magnitude greater than any membrane gradient found in animals. It seems surprising, therefore, that there have been only a handful of papers relating oxidative stress in plants with cytosolic calcium concentration.

EGTA protects protoplasts from oxidative cell death

The rate of cell death when barley mesophyll protoplasts are treated with either 0.1 mM paraquat in the light or 5 mM hydrogen peroxide is dramatically reduced if 2 mM EGTA is included in the suspension medium [35]. As EGTA will chelate extracellular calcium, it should prevent stress-induced calcium influx. It appears possible that cell death may be due, at least in part, to calcium influx.

Plant Ca²⁺-ATPase is sensitive to oxidative stress

Paraquat treatment (which causes superoxide production in the chloroplast [36]) of pea seedlings has shown that the plasma-membrane Ca²⁺-ATPase is highly sensitive to oxidative stress [37]. Paraquat (0.1 mM) had no or very little effect on the soluble enzymes malate dehydrogenase (mitochondrial, chloroplastic and cytoplasmic), triosephosphate isomerase (chloroplastic and mitochondrial) and hydroxypyruvate reductase (peroxisomal). Ca²⁺-ATPase was inhibited by 44% by 0.1 mM paraquat and by 75% by 0.3 mM paraquat, whereas Mg²⁺-ATPase was inhibited by only 9% and 13%, respectively, by these treatments. Ca²⁺-ATPase from plants appears to be highly sensitive to oxidizing conditions, as has been shown in some animal systems [18,29,30].

Effect of ozone on calcium distribution

Using membrane vesicles isolated from pinto beans exposed to ozone, it has been shown that...
passive influx of calcium into cells is increased by ozone, and that subsequent inability to remove calcium from the cell is involved in cell death [38].

Studies of calcium distribution in spruce [39] and pine [40] needles result in similar conclusions. There is evidence of increased calcium oxalate crystallization, reflecting an increase in the influx of calcium across the plasma membrane. The observed increase in callose and cellulose formation in ozone-fumigated cells is thought to be a result of calcium activation of polysaccharide synthesis [39].

Single-cell sampling using a micropressure probe allows elemental analysis of single cell vacuoles [34,41,42]. In epidermal cells of barley leaves, vacuolar calcium concentrations in the region of 25–45 mM can be observed (Figures 1a and 1b). When plants are exposed to 70 p.p.b. ozone for 8 h day, every other day, the epidermal cells contain between 70% and 200% more calcium (Figures 1a and 1b) depending on the position on the leaf and which leaf surface the samples are taken from. Ozone does not have a significant effect on potassium (Figures 1c and 1d). This increased calcium accumulation cannot be due to elevated transport of calcium into the leaf through transpiration, as ozone consistently reduces stomatal conductance in this experiment (results not shown). Taken together, there is powerful evidence that ozone treatment does indeed cause an increase in calcium influx into the plant cell.

**Oxidative stress closes stomata**

The closure of stomata owing to external stimuli such as abscisic acid or CO₂ has been shown to involve a calcium signalling event (characterized as an increase in cytosolic calcium) that triggers

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**Figure 1**

The concentrations of calcium (a and b) and potassium (c and d) of vacuolar sap from single upper (a and c) and lower (b and d) epidermal cells from ozone-fumigated barley (shaded) compared with control, unfumigated plants (open).

Bars represent standard errors and n = 9. The barley plants received Purafil and charcoal-filtered air (control) or filtered air plus 70 p.p.b. ozone for 8 h per day every other day from germination. The plants also received 250 μE light for 16 h/day, 25°C day, 20°C night, and the third leaf was sampled on the day it reached full expansion. Cell sap was extracted using the pressure probe and metals were measured using EDAX as previously described [41,42]. Similar results were obtained in a repeat experiment (data not shown).
Response of cytosolic calcium concentration to 10 mM hydrogen peroxide treatment (arrow) in apoaequorin-transformed *Arabidopsis* using the same methods as previously published [47].

Cytosolic free calcium was calibrated using standard methods [47] and a calibration curve determined using the specific apoaequorin isoform used for transformation [48].

![Graph](image)

intracellular ion movements, which in turn cause turgor loss [10,43,44]. The observation that paraquat or hydrogen peroxide treatment of illuminated epidermal strips causes stomatal closure that is partially prevented by calcium chelators or antagonists [45] provides indirect evidence that oxidative stress does indeed elevate plant cell cytosolic calcium concentrations.

**Apoaequorin-transformed plants reveal oxidative calcium signals**

The best evidence to date of oxidative stress-induced alterations in calcium homoeostasis comes from work using tobacco plants transformed with the jellyfish gene encoding apoaequorin [46]. These plants contain the jellyfish protein in their cytosol. When combined with the chromophore coelenterazine, the resultant protein, aequorin, directly reports calcium concentration by the emission of blue light [9].

When tobacco plants containing aequorin were treated with sublethal concentrations of hydrogen peroxide, a transient luminescence response was observed. Control experiments confirmed that this reflected elevated cytosolic calcium concentration. It was shown that the magnitude of the transient calcium elevation was dose dependent, being detectable with as little as 0.05 mM hydrogen peroxide. It was also shown that several hours were required before a plant previously triggered by hydrogen peroxide could be made to respond in a similar manner a second time, although the plants' ability to respond luminescently to cold stimuli was unaffected by hydrogen peroxide. It was concluded that there is a calcium-dependent signalling process that responds to hydrogen peroxide and which is unrelated to calcium signalling processes associated with cold or touch stimuli.

It is now possible to calibrate the hydrogen peroxide-induced calcium signal using apoaequorin-transformed *Arabidopsis* (Figure 2). Hydrogen peroxide (10 mM) causes a transient elevation of cytosolic calcium from 100 nM (resting) to 500 nM.

The use of inhibitors in the tobacco study implicated the oxidation state of the glutathione pool in the regulation of the calcium signal. Plants pretreated with the glutathione synthesis inhibitor buthionine sulfoximine showed increased calcium response. This would be expected, as the smaller glutathione pool will be more rapidly oxidized. On the other hand, inhibitors of ascorbate peroxidase decreased the height of the calcium response, but the response was maintained for longer. Reaction of ascorbate with hydrogen peroxide catalysed by ascorbate peroxidase should promote the oxidation of glutathione via dehydroascorbate reductase. Inhibition of ascorbate peroxidase will slow glutathione oxidation. This implies that oxidation of the glutathione pool precedes and precipitates the calcium signal.

**Conclusions**

The abundance of papers in animal systems combined with the slowly growing number of studies in plants provide powerful evidence that a primary consequence of oxidative stress in plants is a perturbation of cytosolic calcium concentration. This has two profound implications. Firstly, altered activity of cellular calcium under oxidative stress could be a highly developed system for perception and response to oxidative stress. Secondly, as uncontrolled elevation of cytosolic calcium will have major effects on metabolism, the pathology of oxidative stress cannot be divorced from the pathology of calcium.

Free Radical Processes in Plants


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