COMMUNICATION

Calcium in Lipid Peroxidation: Does Calcium Interact with Superoxide?

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Using a pulse radiolysis approach to generate and observe superoxide anions (O$_2^-$) in the absence and presence of calcium, we have attempted to verify the recent hypothesis of Babizhayev (Arch. Biochem. Biophys. 266, 446–451, 1988) of a Ca$^{2+}$–O$_2^-$ interaction during lipid peroxidation. We could not observe rapid scavenging of O$_2^-$ or complex formation with Ca$^{2+}$ to account for an inhibitory effect of this cation on lipid peroxidation. Neither could we agree that the stimulatory effect is due to liberation of catalytic ferrous iron from weak complexes by Ca$^{2+}$. Drawing on reports in the literature, we propose an alternate explanation for the apparent stimulation of lipid peroxidation by low Ca$^{2+}$ concentrations. In our view, this is not a direct effect, but reflects independently initiated processes of lipid peroxidation and Ca$^{2+}$ translocation, which interact subsequently in a synergistic manner. The reported inhibition at high Ca$^{2+}$ concentrations is considered an artifact as it was observed at levels far in excess of those relevant to animal systems (but not necessarily in some plant compartments).

The ubiquity of calcium and its participation in numerous cell regulatory processes by mere changes in its concentration gradient have led to its recognition as an important second messenger substance in both animals (1, 2) and plants (3, 4). As determinations of Ca$^{2+}$ concentrations in plants are far more difficult (3), the following values at present pertain only to animal systems (but not necessarily in some plant compartments).

Recently, Babizhayev (12) has attempted to explain the disparate effects of low (stimulation) and high (inhibition) concentration of Ca$^{2+}$ on lipid peroxidation. He used xanthine/xanthine oxidase and NADH/phenazine methosulfate as radical sources to initiate lipid peroxidation in conjunction with the nitroblue tetrazolium assay for O$_2^-$. Because these generation procedures and this assay can be rather unspecific for O$_2^-$, we decided to reinvestigate the question of Ca$^{2+}$/O$_2^-$ interaction using the pulse radiolysis technique as a highly specific means for O$_2^-$ generation and detection. We addressed the two major points of the hypothesis:

(i) Is there any spectral evidence for a Ca$^{2+}$–O$_2^-$ complex?
(ii) Does Ca$^{2+}$ alter the rate of O$_2^-$ decay?

We found that neither of these points could be substantiated, in line with an earlier pulse radiolysis and EPR study on the interaction between Ca$^{2+}$ and O$_2^-$ (13). On the basis of these data and an extensive literature search on various aspects of Ca$^{2+}$ metabolism, we have come to alternate conclusions on the interrelationship of calcium and lipid peroxidation.

MATERIALS AND METHODS

Calcium acetate, sodium formate, diethylene triamine pentaacetic acid (DTPA)$^1$ and the disodium salt of ethylene diamine tetraacetic acid (EDTA) were of analytical grade and used as supplied; solutions were prepared with "Milli-Q" water and buffered with phosphate (0.1 M) at pH 8.4 or adjusted with NaOH to pH 10.4. Ca$^{2+}$ concentrations were between 0.001 and 1 mM and those of EDTA or DTPA at 0.1 mM each.

Pulse radiolysis was performed with an instrumental setup described earlier (14). Electron pulses (100 ns, 1.7 MeV) from a Bectron 705 accelerator (Hewlett-Packard) were delivered into an aqueous oxygen-saturated solution containing 10 mM sodium formate. Under these conditions all primary radicals—hydrated electrons, hydroxyl radicals, and hydrogen atoms—are converted into O$_2^-$, yielding this radical species exclusively (about 20 μM at a pulse dose of 30 Gy; Ref. (15)). The absorption of O$_2^-$ was observed at 250 nm (ε = 2350 M$^{-1}$ cm$^{-1}$, Ref. (16)) and the second-order decay rates were obtained from linearized regression analyses. Kinetic modeling by superposition of presumptive pseudo-first-order reactions allowed the determination of the upper limits, at which these reactions would show an acceleration.

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$^1$ Abbreviation used: DTPA, diethylene triamine pentaacetic acid.
RESULTS

The dose-normalized spectra of O₂ at pH 10.4 in the absence of Ca²⁺ and with Ca²⁺ concentrations of 0.05–1 mM were identical within experimental error (Fig. 1). Despite the larger scatter in the data for the spectra at the final observation time of 3.38 s this scatter was not due to any concentration-dependent trends. In all cases, the decay kinetics of O₂ are unchanged and remain a second-order process even at the maximal concentration of Ca²⁺.

We also observed no difference in the decay kinetics of O₂ in the absence or presence of 0.1 mM Ca³⁺ and 0.1 mM of either EDTA or DTPA—demonstrating the lack of effect of adventitious transition metals under our pulse-radiolytic conditions (data not shown).

Calculations of the pseudo-first-order contributions to the overall decay of O₂ by a second-order process (spontaneous dismutation) gives an upper limit for the reaction rate constant of Ca²⁺ with O₂ of 500 M⁻¹s⁻¹ (Fig. 2). Because of the high turbidity of the phosphate-buffered solutions containing >0.5 mM Ca²⁺, these signals are affected by considerable noise levels. As a consequence, computer fits resulting in values around 100 M⁻¹s⁻¹ (see Discussion) are practically indistinguishable from control experiments in the absence of Ca²⁺. Therefore our detection limit is defined as the minimal rate constant at which a deviation from the experimental results is readily apparent. The decay rate constant of 5 × 10⁴ M⁻¹s⁻¹, as reported by Babizhayev (12), would produce an apparent decay much faster than was observed experimentally (see Fig. 2).

DISCUSSION

Using pulse radiolysis, a highly specific means to generate and directly observe superoxide anions, we are not able to corroborate either the formation of a Ca²⁺-O₂ complex or a relatively fast reaction of O₂ with Ca²⁺ as reported by Babizhayev (12). These two reactions would result in opposite effects on the decay kinetics of O₂: (i) retardation in case of complex formation (as was the interpretation by Westerman (17) of the results of Bray et al., Ref. (13)) and (ii) acceleration in case of scavenging. Incidentally, according to the latest Radiation Chemistry Data Center list on rate constants of the primary radicals (19), Ca²⁺ does not scavenge any of these radicals.

In their pulse radiolysis study, Bray et al. (13) used CaCl₂ concentrations of 0.25 M in Hepes buffer (10 mM, pH 6.8–7.2) and EDTA (0.1 mM). They found a (pseudo) first-order decay of O₂ with a half-life of about 41.5 ms, which is equivalent to a second-order rate constant of about 10 M⁻¹s⁻¹—a value surprisingly close to our computer fits, which we neglected in favor of a clearly defined detection limit (see above). Bray et al. (13) also found that Ca²⁺ (at concentrations as low as 0.3 mM) caused some alterations in the EPR spectrum of O₂, but they did not attribute these changes to complex formation. Their results are in complete agreement with our experiments which failed to reveal such a complex spectroscopically or kinetically, at Ca²⁺ concentrations up to 1 mM, the maximal physiological level in animal systems (1, 2). Thus, the proposal of Babizhayev (12) that Ca²⁺ inhibits lipid peroxidation at high concentrations either due to complex formation with O₂ or by scavenging of O₂ cannot be supported. We can only assume that his high rate constants arise from the use of both unspecific sources of O₂ (19, 20) and an unspecific assay (21, 22), problems which have previously been encountered and have been commented on (23–25).

What about Babizhayev’s proposal, that calcium stimulates lipid peroxidation at low concentrations by replacing and releasing oxidation-promoting Fe²⁺ from complexes with fatty acids and/or phospholipids? His arguments are actually based on early work by Gutteridge (26), who used Ca²⁺ concentrations as high as 0.1 M. The interactions of fatty acids and phospholipids with calcium were first studied by Serhan et al. (27) and later by Kim and LaBella (28), both groups finding that only oxidized fatty acids (especially derivatives of linoleic and arachidonic acids) and phospholipids (but not phosphatidylcholine) are capable of translocating Ca²⁺ across liposomal membranes or into the lipophilic phase of a two-phase parti-
tion model. As complexes of Ca$^{2+}$ with nonoxidized fatty acids would be very weak—if they existed at all—the explanation by Babizhayev (12) for the 1:1 stoichiometry as replacement of Fe$^{3+}$ by Ca$^{2+}$ in such complexes is unlikely.

Because Babizhayev's arguments appear to be flawed, are there reasonable explanations for the observed disparate effects of Ca$^{2+}$ concentration on lipid peroxidation (26, 29)? As it turns out, inhibition of lipid peroxidation by high calcium concentrations has only been observed at Ca$^{2+}$ levels above 1 mM in liposomes (20) and in heart mitochondria and microsomes (29). In our view, this has little relevance to physiological conditions, i.e., concentrations below 10 μM. While we believe that the inhibitory effect of high Ca$^{2+}$ concentrations on lipid peroxidation is an artifact, it has to be kept in mind that plants may contain higher levels of free Ca$^{2+}$ in their cell walls (30).

Stimulation of lipid peroxidation by low Ca$^{2+}$ concentrations, however, appears to be genuine because it has been observed under a wide variety of conditions (6, 8, 31-35). Furthermore, the concentrations used during these experiments approach intracellular levels (<10 μM) and are thus quite pertinent to physiological regulatory processes. Despite the fact that a considerable number of enzymatic or biological processes are known to be affected by Ca$^{2+}$ (2), there are no likely candidates for a direct initiation reaction of lipid peroxidation by calcium. Therefore, using the evidence that (i) oxidized fatty acids promote calcium transport (27, 28, 29, 32), (ii) phospholipase A$_2$ is activated by calcium (33, 35, 38) or possibly by oxygen-derived radicals (39), (iii) this enzyme acts preferentially on peroxidized lipids (40), (iv) the passive calcium influx is enhanced by collapse of the membrane potential (41-43), and (v) Ca$^{2+}$-ATPase is inhibited by lipid peroxidation (35, 44-47) or reactive oxygen species (8, 48, 49), we propose that:

(i) The initial reactions leading to lipid peroxidation are not dependent on any changes of the Ca$^{2+}$ gradient. The temporary sequences of oxidant injury, activation of DNA repair and—more slowly—increase of intracellular calcium have been most thoroughly investigated in lung cells (50).

(ii) Following this initiation reaction, the action of phospholipase A$_2$ and the stimulation of its activity by Ca$^{2+}$ (33, 35) may act in combination, suggesting an apparent stimulating effect.

(iii) The controlled Ca$^{2+}$ translocation via Ca$^{2+}$-ATPase (51, 52) becomes imperative due to inactivation of the enzyme (35, 44-47), probably by oxidation of critical thiol groups (53) after GSH depletion (8).

(iv) The increased Ca$^{2+}$ influx observed after initiation of lipid peroxidation (35, 41-46, 54, 55) may be the result of an interaction of Ca$^{2+}$ with oxidized fatty acids (27, 28) and can likewise be seen as 'stimulation.'

In conclusion, we found no evidence for any 'inhibitory effect' of calcium on lipid peroxidation via scavenging or complexing of O$_2^-$. As pertains to the apparent stimulatory effect, we propose that this is actually the result of an independently initiated lipid peroxidation process that acts synergistically with Ca$^{2+}$ translocation.

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