ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 278, No. 1, April, pp. 269–272, 1990

# COMMUNICATION

## Calcium in Lipid Peroxidation: Does Calcium Interact with Superoxide?

Wolf Bors,* Garry R. Buettner,† Christa Michel,* and Manfred Saran*
*Institute für Strahlenbiologie, GSF Research Center, D-8042 Neuherberg, FRG; and †ESR Center, EMRB 68, University of Iowa, Iowa City, Iowa 52242

Received September 22, 1989, and in revised form November 1, 1989

**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. The levels of $Ca^{2+}$ are usually about 1 mM in extracellular space and about 10 nM in the cytoplasm, reaching 10 μM when import systems override the export mechanisms (1, 2). In the last few years a correlation of $Ca^{2+}$ levels and oxygen activation has been discovered, with the majority of reports concerning activation of neutrophils to generate superoxide anions (3). In contrast, the interrelationship of calcium and lipid peroxidation in either animals (6–8) or plants (9–11) has received less attention, yet it would be quite important were a direct effect of calcium proven.

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 $Ca^{2+} \cdot O_2^-$ 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 Febetron 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 ($\epsilon = 2350\ M^{-1}\ cm^{-1}$, 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.

[1] Abbreviation used: DTPA, diethylene triamine pentaacetic acid.

0003-9861/90 $3.00

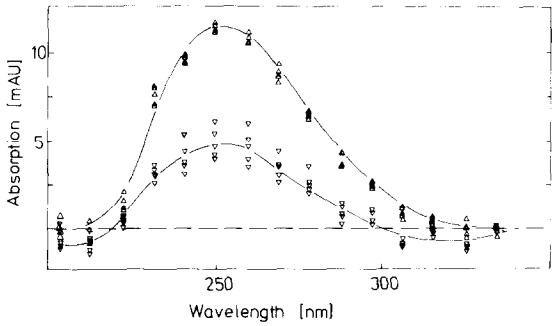


**FIG. 1.** Transient spectra of $O_2^-$ in the absence and presence of $Ca^{2+}$. Oxygen-saturated aqueous solutions containing 10 mM sodium formate and 0, 0.05, 0.1, 0.5, and 1.0 mM calcium acetate, pH 10.4 adjusted with NaOH. Dose-normalized spectra, average pulse dose for all experiments 39 Gy (each spectrum is the composite of four to six individual pulses). (△) Initial observation time 28 ms; (▽) final observation time 3.38 s (downward sequence of transient spectra: 1.0, 0.05, 0.1, 0, 0.5 mM $Ca^{2+}$).

## RESULTS

The dose-normalized spectra of $O_2^-$ at pH 10.4 in the absence of $Ca^{2+}$ and with $Ca^{2+}$ 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 $O_2^-$ are unchanged and remain a second-order process even at the maximal concentration of $Ca^{2+}$.

We also observed no difference in the decay kinetics of $O_2^-$ in the absence or presence of 1 mM $Ca^{2+}$ 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 $O_2^-$ by a second-order process (spontaneous dismutation) gives an upper limit for the reaction rate constant of $Ca^{2+}$ with $O_2^-$ of 500 $M^{-1}s^{-1}$ (Fig. 2). Because of the high turbidity of the phosphate-buffered solutions containing >0.5 mM $Ca^{2+}$, these signals are affected by considerable noise levels. As a consequence, computer fits resulting in values around 70 $M^{-1}s^{-1}$ (see Discussion) are practically indistinguishable from control experiments in the absence of $Ca^{2+}$. 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 $5 \times 10^4$ $M^{-1}s^{-1}$, 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 $Ca^{2+} \cdot O_2^-$ complex or a relatively fast reaction of $O_2^-$ with $Ca^{2+}$ as reported by Babizhayev (12). These two reactions would result in opposite effects on the decay kinetics of $O_2^-$: (i) retardation in case of complex formation (as was the interpretation by Westermark (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 (18), $Ca^{2+}$ does not scavenge any of these radicals.

In their pulse radiolysis study, Bray *et al.* (13) used $CaCl_2$ concentrations of 0.25 M in Hepes buffer (10 mM, pH 6.8–7.2) and EDTA (0.5 mM). They found a (pseudo) first-order decay of $O_2^-$ with a half-life of about 41.5 ms, which is equivalent to a second-order rate constant of about 70 $M^{-1}s^{-1}$—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 $Ca^{2+}$ (at concentrations as low as 0.3 mM) caused some alterations in the EPR spectrum of $O_2^-$, 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 $Ca^{2+}$ concentrations up to 1 mM, the maximal physiological level in animal systems (1, 2). Thus, the proposal of Babizhayev (12) that $Ca^{2+}$ inhibits lipid peroxidation at high concentrations either due to complex formation with $O_2^-$ or by scavenging of $O_2^-$ cannot be supported. We can only assume that his high rate constants arise from the use of both *unspecific* sources of $O_2^-$ (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 $Fe^{2+}$ from complexes with fatty acids and/or phospholipids? His arguments are actually based on early work by Gutteridge (26), who used $Ca^{2+}$ 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 $Ca^{2+}$ across liposomal membranes or into the lipophilic phase of a two-phase parti-

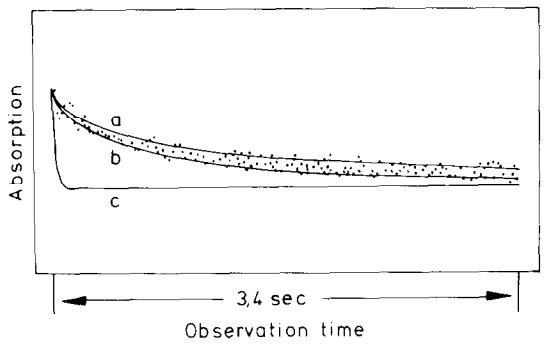


**FIG. 2.** Kinetic traces of experimental and calculated $O_2^-$ decay. Aqueous solutions, buffered with phosphate (0.1 M) at pH 8.4, containing sodium formate (10 mM), calcium acetate (1 mM), EDTA (0.1 mM) and saturated with oxygen; a pulse dose of 33 Gy results in an initial $O_2^-$ yield of 22 μM. Abscissa, observation time after the pulse (total 3.44 s); ordinate, absorption changes of $O_2^-$ at 250 nm (arbitrary scale). (a) Calculated curve, assuming lack of influence of $Ca^{2+}$ on $O_2^-$ decay; (b) experimental curve, consisting of the second-order decay of $O_2^-$ and a superimposed contribution of $Ca^{2+}$ reacting with $O_2^-$ (detection limit for the latter reaction is 500 $M^{-1}s^{-1}$); (c) calculated decay curve based on the rate constant of $Ca^{2+} + O_2^-$ of $5 \times 10^4$ $M^{-1}s^{-1}$, as reported by Babizhayev (12).

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 $Fe^{2+}$ 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 $\mu$M. 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 $\mu$M) and are thus quite pertinent to biological 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, 36, 37), (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 inoperative due to inactivation of the enzyme (35, 44–49), 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.

## ACKNOWLEDGMENTS

We appreciate the stimulating discussions with Werner Heller, Silvio Leonardi, and Christian Langebartels, BIOP (GSF). Alf Kruse was responsible for the pulse radiolysis experiments and Matthias Born and Beate Kreileder helped with the manuscript.

## REFERENCES

1. Kleinig, H., and Sitte, P. (1986) Zellbiologie, 2. Auflage, p. 115, G. Fischer, Stuttgart.
2. Yagi, K., and Miyazaki, T. (1988) Calcium Signal and the Cell Response, Japan Sci. Soc. Press, Tokyo/Springer, Berlin.
3. Kauss, H. (1987) *Annu. Rev. Plant Physiol.* **38,** 47–72.
4. Morse, M. J., Satter, R. L., Crain, R. C., and Cote, G. G. (1989) *Physiol. Plant.* **76,** 118–121.
5. Badwey, J. A., and Karnovsky, M. L. (1986) *Curr. Top. Cell. Regul.* **28,** 183–208.
6. Braughler, J. M. (1988) *in* Oxygen Radicals and Tissue Injury (Halliwell, B., Ed.), pp. 99–106, Upjohn Co., Kalamazoo, MI.
7. Pascoe, G. A., and Reed, D. J. (1989) *Free Rad. Biol. Med.* **6,** 209–224.
8. Mirabelli, F., Salis, A., Vairetti, M., Bellomó, G., Thor, H., and Orrenius, S. (1989) *Arch. Biochem. Biophys.* **270,** 478–488.
9. Dhindsa, R. S., Plumb-Dhindsa, P. L., and Reid, D. M. (1982) *Physiol. Plant.* **56,** 453–457.
10. Leshem, Y. Y., Sridhara, S., and Thompson, J. E. (1984) *Plant Physiol.* **75,** 329–335.
11. Belver, A., Roldan, M., Rodriguez, M. P., and Donaire, J. P. (1988) *Grasas Aceites* **39,** 182–184.
12. Babizhayev, M. A. (1988) *Arch. Biochem. Biophys.* **266,** 446–451.
13. Bray, R. C., Mautner, G. N., Fielden, E. M., and Carle, C. I. (1977) *in* Superoxide and Superoxide Dismutases (Michelson, A. M., McCord, J. M., and Fridovich, I., Eds.), pp. 62–76, Academic Press, London.
14. Saran, M., Vetter, G., Erben-Russ, M., Winter, R., Kruse, A., Michel, C., and Bors, W. (1987) *Rev. Sci. Instrum.* **58,** 363–368.
15. Bors, W., Saran, M., Michel, C., and Tait, D. (1984) *in* Advances on Oxygen Radicals and Radioprotectors (Breccia, A., Greenstock, C. L., and Tamba, M., Eds.), pp. 13–27, Lo Scarabeo Ed. Sci., Bologna.
16. Bielski, B. H. J. (1978) *Photochem. Photobiol.* **28,** 645–649.
17. Westermark, U. (1982) *Wood Sci. Technol.* **16,** 71–78.
18. Buxton, G. V., Greenstock, C. L., Helman, W. P., and Ross, A. B. (1988) *J. Phys. Chem. Ref. Data* **17,** 513–904.
19. Fridovich, I. (1985) *in* CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., Ed.), pp. 51–53, CRC Press, Boca Raton.
20. Richter, H. W., Fetrow, M. A., Lewis, R. E., and Waddell, W. H. (1982) *J. Amer. Chem. Soc.* **104,** 1667–1671.
21. Bielski, B. H. J., Shiue, G. G., and Bajuk, S. (1980) *J. Phys. Chem.* **84,** 830–833.
22. Auclair, C., and Voisin, E. (1985) *in* CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., Ed.), pp. 123–132, CRC Press, Boca Raton.
23. Bors, W., Michel, C., and Saran, M. (1979) *Eur. J. Biochem.* **95,** 621–627.
24. Bors, W., Saran, M., and Michel, C. (1982) *in* Superoxide Dismutase (Oberley, L. W., Ed.), Vol. II, pp. 31–62, CRC Press, Boca Raton.
25. Winterbourn, C. C., and Sutton, H. C. (1986) *Arch. Biochem. Biophys.* **244,** 27–34.
26. Gutteridge, J. M. C. (1977) *Biochem. Biophys. Res. Commun.* **74,** 529–536.
27. Serhan, C., Anderson, P., Goodman, E., Dunham, P., and Weissman, G. (1981) *J. Biol. Chem.* **256,** 2736–2741.

28. Kim, R. S., and LaBella, F. S. (1985) *Biochim. Biophys. Acta* **833,** 386–395.
29. Kagan, V. E., Savov, V. M., Didenko, V. V., Arkhipenko, Y. V., and Meerson, F. Z. (1983) *Bull. Exp. Biol. Med.* **95,** 458–461.
30. Thomas, F. M., and Runge, M. (1988) *Z. Pflanzenernähr. Bodenk.* **151,** 211–217.
31. Jain, S. K., and Shohet, S. B. (1981) *Biochim. Biophys. Acta* **642,** 46–54.
32. Perry, G., and Epel, D. (1985) *Dev. Biol.* **107,** 47–57.
33. Beatrice, M. C., Stiers, D. L., and Pfeiffer, D. R. (1984) *J. Biol. Chem.* **259,** 1279–1287.
34. Richter, C., and Frei, B. (1986) *in* Oxidative Stress (Sies, H., Ed.), pp. 221–241, Academic Press, London.
35. Malis, C. D., and Bonventre, J. V. (1986) *J. Biol. Chem.* **261,** 14,201–14,208.
36. Lebedev, A. V., Levitsky, D. O., and Loginov, V. A. (1982) *in* Advances in Myocardiology (Chazov, E., Smirnov, V., and Dhatta, N. S., Eds.), Vol. 3, pp. 425–438, Plenum, New York.
37. Kim, R. S., and LaBella, F. S. (1988) *J. Mol. Cell. Cardiol.* **20,** 119–130.
38. Nishida, T., Inoue, T., Kamiike, W., Kawashima, Y., and Tagawa, K. (1989) *J. Biochem.* **106,** 533–538.
39. Au, A. M., Chan, P. H., and Fishman, R. A. (1985) *J. Cell. Biochem.* **27,** 449–453.
40. van Kuijk, F. J. G. M., Sevanian, A., Handelman, G. J., and Dratz, E. A. (1987) *Trends Biochem. Sci.* **12,** 31–34.
41. Cadenas, E., and Boveris, A. (1980) *Biochem. J.* **188,** 31–37.
42. Palmer, J. W., and Pfeiffer, D. R. (1981) *J. Biol. Chem.* **256,** 6742–6750.
43. Mirabelli, F., Richelmi, P., Salis, A., Marinoni, V., Bianchi, A., Berte, F., and Bellomo, G. (1987) *Medec. Biol. Environ.* **15,** 313–321.
44. Kim, R. S., Sukhu, B., and LaBella, F. S. (1988) *Biochim. Biophys. Acta* **961,** 270–277.
45. Kukreja, R. C., Okabe, E., Schrier, G. M., and Hess, M. L. (1988) *Arch. Biochem. Biophys.* **261,** 447–457.
46. Kukreja, R. C., Weaver, A. B., and Hess, M. L. (1989) *Biochim. Biophys. Acta* **990,** 198–205.
47. Ohta, A., Mohri, T., and Ohyashiki, T. (1989) *Biochim. Biophys. Acta* **984,** 151–157.
48. Kaneko, M., Beamish, R. E., and Dhalla, N. S. (1989) *Amer. J. Physiol.* **256,** H368–H374.
49. Grover, A. K., and Samson, S. E. (1989) *Amer. J. Physiol.* **256,** C666–C673.
50. Schraufstaetter, I. U., Hyslop, P. A., Jackson, J., Revak, S. D., and Cochrane, C. C. (1987) *Bull. Eur. Physiopathol. Respir.* **23,** 297–302.
51. Inesi, G. (1982) *Annu. Rev. Physiol.* **44,** 573–601.
52. Schatzmann, H. J. (1989) *Annu. Rev. Physiol.* **51,** 473–485.
53. Kison, R., Meyer, H. E., and Schoner, W. (1989) *Eur. J. Biochem.* **181,** 503–511.
54. Novgorodov, S. A., Gogvadze, V. G., Medvedev, B. I., and Zinchenko, V. P. (1989) *FEBS Lett.* **248,** 179–181.
55. Levitsky, D. O., Lebedev, A. V., Kuzmin, A. V., and Brovkovich, V. M. (1988) *in* Role of Oxygen Radicals in Cardiovascular Diseases (L'Abbate, A., and Ursini, F., Eds.), pp. 127–142, Kluwer Academic, Doordrecht.