THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 6, Issue of February 9, pp. 2929–2934, 1996

Printed in U.S.A.

Effect of Inorganic Phosphate Concentration on the Nature of Inner Mitochondrial Membrane Alterations Mediated by Ca^{2+} Ions

A PROPOSED MODEL FOR PHOSPHATE-STIMULATED LIPID PEROXIDATION*

(Received for publication, July 21, 1995, and in revised form, November 22, 1995)

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Addition of high concentrations (>1 mM) of inorganic phosphate (P_i) or arsenate to Ca^{2+}-loaded mitochondria was followed by increased rates of H_2O_2 production, membrane lipid peroxidation, and swelling. Mitochondrial swelling was only partially prevented either by butylhydroxytoluene, an inhibitor of lipid peroxidation, or cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. This swelling was totally prevented by the simultaneous presence of these compounds. At lower P_i concentrations (1 mM), mitochondrial swelling is reversible and prevented by cyclosporin A, but not by butylhydroxytoluene. In any case (low or high phosphate concentration) exogenous catalase prevented mitochondrial swelling, suggesting that reactive oxygen species (ROS) participate in these mechanisms. Altogether, the data suggest that, at low P_i concentrations, membrane permeabilization is reversible and mediated by opening of the mitochondrial permeability transition pore, whereas at high P_i concentrations, membrane permeabilization is irreversible because lipid peroxidation also takes place. Under these conditions, lipid peroxidation is strongly inhibited by sorbate, a putative quencher of triplet carbonyl species. This suggests that high P_i or arsenate concentrations stimulate propagation of the peroxidative reactions initiated by mitochondrial-generated ROS because these anions are able to catalyze C_n-aldehyde tautomerization producing enols, which can be oxidized by hemeproteins to yield the lower C_{n-1}-aldehyde in the triplet state. This proposition was also supported by experiments using a model system consisting of phosphatidylcholine/dicethylphosphate liposomes and the triplet acetone-generating system isobutanal/horseradish peroxidase, where phosphate and Ca^{2+} cooperate to increase the yield of thiobarbituric acid-reactive substances.

The decrease in ATP levels that occurs under pathological conditions such as prolonged anoxia or ischemia/reperfusion results in inorganic phosphate (P_i) accumulation and alterations in intracellular Ca^{2+} homeostasis (1–7). It is argued that high P_i concentrations mimic the metabolic conditions prevalent during ischemia and that, at concentrations higher than 10 mM, P_i inhibits mitochondrial oxidative phosphorylation (8). Indeed, it is well known (see Refs. 9 and 10, and references therein) that accumulation of Ca^{2+} and P_i by mitochondria results in increased permeability of the inner mitochondrial membrane. This is proposed to be a key step in the pathogenesis of cell injury that occurs during ischemia and reperfusion (8–11). Different mechanisms such as lipid peroxidation, phospholipid hydrolysis by phospholipase A_2, or opening of the mitochondrial permeability transition pore (MTP)[1] have been proposed to take place under these conditions (9–14). In this regard, P_i concentration, which is reported to increase up to 20 mM in cells exposed to prolonged anoxia (6), has a profound effect in the rate, extent, and nature of mitochondrial membrane alterations caused by Ca^{2+} ions (8–11, 14–16).

Data from our laboratory (17–20) provided evidence that in the absence of added P_i, the membrane alterations caused by Ca^{2+} seem to be mediated mainly by the attack of mitochondrial generated reactive oxygen radicals (ROS) to membrane proteins, resulting in opening of the MTP, a mechanism sensitive to cyclosporin A (9, 10). This process is stimulated by prooxidants such as *t*-butyl hydroperoxide or diamide (17, 19, 21), which exhaust mitochondrial reducing power such as NAD(P)H and GSH (9, 22). Under these conditions, the rate of lipid peroxidation is low and the membrane can be quickly resealed by the addition of EGTA and disulfide reductants (17, 21). In contrast, lipid peroxidation is the main form of irreversible mitochondrial membrane permeabilization caused by Ca^{2+} in the presence of other oxidants such as Fe(II)citrate or Fe(II)ATP (23, 24). Interestingly, other data (18, 20) from our laboratory indicate that, while the reduced state of coenzyme Q stimulates the protein oxidation that occurs in the presence of Ca^{2+} and *t*-butyl hydroperoxide, it protects against damage that occurs in the presence of Ca^{2+} and Fe(II)citrate. This supports the notion that mitochondrial membrane alterations caused by Ca^{2+} in situations of oxidative stress may occur via protein thiol oxidation and/or lipid peroxidation.

The aim of this study was to investigate the mechanisms underlying the stimulation of Ca^{2+}-mediated mitochondrial inner membrane permeabilization by different P_i concentrations. Phosphate is the earliest "inducing agent" described (see

* This work was partially supported by grants from the Brazilian Agencies Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa (CNPq), Programa de Apoio ao Desenvolvimento Científico e Tecnológico, Financiadora de Estudos e Projetos, and Fundação de Amparo ao Ensino e Pesquisa. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by a CNPq scholarship.
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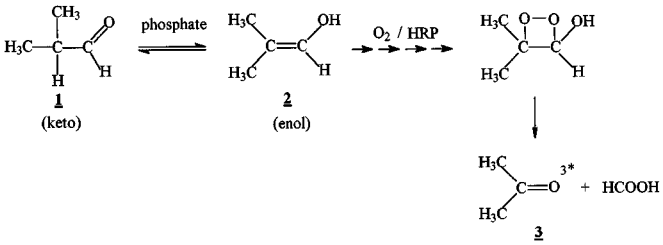
[1] The abbreviations used are: MTP, mitochondrial permeability transition pore; BHT, butylhydroxytoluene; DCP, dicethylphosphate; HRP, horseradish peroxidase; IBAL, isobutanal; PC, phosphatidylcholine; RLM, rat liver mitochondria; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances; PUFA, polyunsaturated fatty acid(s).



SCHEME I. **HRP-catalyzed generation of triplet acetone from isobutanal.**

Refs. 9 and 10, and references therein), and the molecular mechanism by which it mediates membrane permeabilization promoted by Ca^{2+} remains unclear. Phosphate accumulation, which accompanies intracellular Ca^{2+} increase during pathological situations, such as prolonged ischemia and reperfusion (see Ref. 8 and references therein), attests to the relevance of this study. Our hypothesis is that high P_i concentrations and Ca^{2+} act synergistically increasing the extent of irreversible membrane permeabilization, due mainly to aldehyde α-peroxidation, which yields triplet carbonyls able to amplify the reaction chain of the peroxidative process (25). This was approached by examining the effect of Ca^{2+} and phosphate ions on TBARS production by mitochondria and by phosphatidylcholine/dicethylphosphate (PC/DCP) liposomes challenged with isobutanal (IBAL) (**1**) as a phosphate-dependent source of the enol (**2**), which produces triplet acetone (**3**) in the presence of horseradish peroxidase (HPR) (see Scheme I). Mitochondrial swelling and H_2O_2 production were also monitored in Ca^{2+} plus phosphate-challenged mitochondria. Inhibition of TBARS production and mitochondrial swelling by sorbate, a quencher of triplet carbonyls (26), supports the proposition that P_i stimulates lipid peroxidation via aldehyde tautomerization producing enols, very reactive substrates for peroxidases.

MATERIALS AND METHODS

Isolation of Rat Liver Mitochondria—Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA, and 5.0 mM Hepes buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA, and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml. These mitochondria contain 8–10 nmol/mg endogenous calcium as determined by atomic absorption spectroscopy.

Liposome Preparation—PC/DCP liposomes were prepared in deionized water by sonication in a Cole-Parmer ultrasonic homogenizer with a nominal output of 100 watts. After 20 min of sonication (with 1-min interval after each 2-min sonication), the solution was centrifuged at 10,000 × *g* during 20 min to eliminate titanium from the preparation (27).

Standard Incubation Procedure—The experiments were carried out at 30 °C in a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 5.0 μM rotenone, 2 mM succinate, and 10 μM Ca^{2+} as determined by atomic absorption spectroscopy. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments reproducible within 10%.

Oxygen Uptake Measurements—Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a glass chamber equipped with magnetic stirring.

Determination of Mitochondrial Swelling—Mitochondrial swelling was estimated from the decrease in the absorbance at 520 nm measured in a SLM Aminco DW2000 spectrophotometer.

Determination of H_2O_2 Production—H_2O_2 production was determined by the horseradish peroxidase method, as described in Ref. 28.

Thiobarbituric Reactive Substances (TBARS) Measurements—Quantification of TBARS production by mitochondria was performed according to Buege and Aust (29). Briefly, 0.4-ml samples were taken after 10 min of incubation in the conditions described above and mixed with 0.4 ml of 1% TBA in 50 mM NaOH, 0.2 ml of 20% H_3PO_4, and 40 μl of 10 N NaOH. The mixture was heated at 80–90 °C for 15 min in the presence of 1 mM butylhydroxytoluene (BHT). After cooling, 1.5 ml of butanol was added to the solution. The mixture was shaken and centrifuged at 3000 rpm during 5 min. The optical density of the supernatant was determined at 535 nm. In these conditions, the molar extinction coefficient used to calculate TBARS concentrations is 1.56×10^5 M^{-1} cm^{-1}.

Chemicals—All reagents were commercial products of the highest available grade of purity.

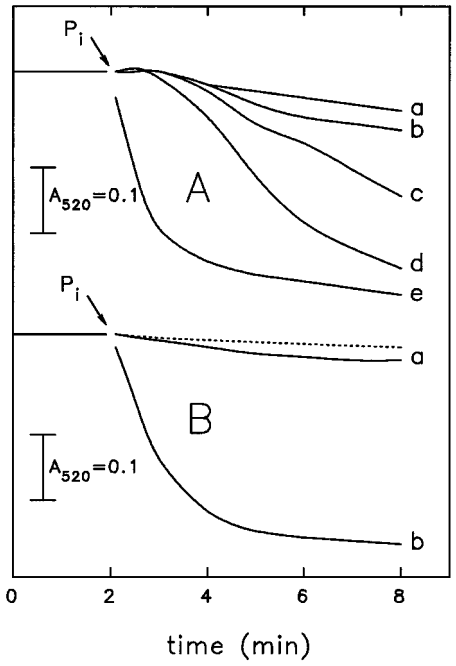


FIG. 1. **Stimulation by P_i of mitochondrial swelling induced by Ca^{2+}.** RLM (0.4 mg/ml) were added to standard reaction medium, containing the following: *panel A*: *a*, 5 μM BHT and 1 μM cyclosporin A; *b*, 2 μM catalase; *c*, 5 μM BHT; *d*, 1 μM cyclosporin A; *e*, no additions; *panel B*: *a*, 1 μM cyclosporin A; *b*, no additions. P_i (4 mM in *panel A*, 1 mM in *panel B*) was added after 2 min of mitochondrial preincubation. The *dashed line* represents a control experiment in the absence of added P_i.

RESULTS

Using the classical mitochondrial swelling technique (9), we showed that addition of 4 mM P_i to a suspension of rat liver mitochondria preincubated (2 min) in reaction medium containing 10 μM Ca^{2+} results in a fast decrease in absorbance of the mitochondrial suspension compatible with extensive mitochondrial swelling (Fig. 1*A*, *line e*). The presence of catalase from the beginning of the experiment caused an almost complete inhibition of mitochondrial swelling (*line b*), suggesting the participation of mitochondrial generated H_2O_2 or H_2O_2-derived radicals in this process (19). In order to ascertain the nature of the membrane alterations observed under these conditions, experiments were performed in presence of the antioxidant BHT, an inhibitor of lipid peroxidation, and of cyclosporin A, an inhibitor of MTP opening. Fig. 1 (*lines c* and *d*) shows that the mitochondrial swelling was partially prevented either by BHT or cyclosporin A, respectively, and totally prevented by the simultaneous presence of BHT and cyclosporin A (*line a*). This behavior is compatible with a membrane permeabilization process mediated partly by lipid peroxidation and partly by MTP opening. In contrast, data in the literature (30–32) indicate that mitochondrial swelling induced by Ca^{2+} in the presence of P_i can be totally prevented by cyclosporin A. Indeed, Fig. 1*B* shows that, under our experimental conditions, at lower P_i concentration (1 mM), cyclosporin A caused an almost complete protection (*line a*) against membrane permeabilization. The *dashed line* represents a control experiment in the absence of added phosphate. In this regard, Fig. 2*A* shows that

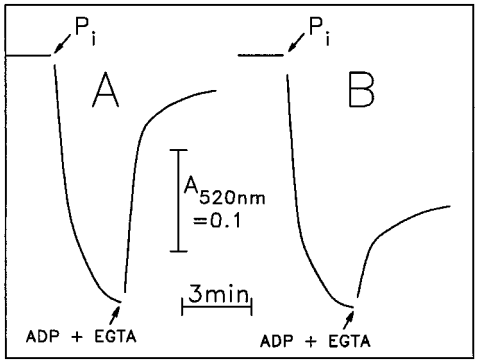


FIG. 2. **Recovery of mitochondrial swelling by addition of EGTA and ADP.** RLM (0.4 mg/ml) were incubated in reaction medium in the absence of sucrose (130 mM KCl, 10 mM Hepes buffer, pH 7.2, 5.0 μM rotenone, 2 mM succinate, and 10 μM Ca^{2+}). P_i (1 mM in *panel A*, 4 mM in *panel B*) was added after 2 min of mitochondrial preincubation. EGTA (1 mM) and 200 μM ADP were added where indicated.

the mitochondrial swelling induced by 1 mM P_i observed in Fig. 1*B* could be reversed almost completely by the addition of EGTA plus ADP, when mitochondria were incubated in a sucrose-free medium. This finding is compatible with membrane resealing (MTP closure), followed by extrusion of the osmotic support that penetrated before addition of EGTA and ADP. At variance with the conditions above, mitochondrial swelling induced by 4 mM P_i was only partially reversed by EGTA and ADP (Fig. 2*B*), as would be expected if, under these conditions, the membrane permeabilization were caused by both MTP opening and lipid peroxidation.

In order to confirm the occurrence of lipid peroxidation during the mitochondrial swelling induced by high P_i concentrations, the production of thiobarbituric acid-reactive substances (TBARS) was measured. Table I shows that TBARS production by mitochondria incubated in the presence of P_i increases with increasing P_i concentrations. This production was greatly diminished when catalase, EGTA, BHT, or sorbate were present. Accordingly, arsenate, a phosphate analog, was also able to cause a great stimulation of TBARS production. In contrast, acetate, which like P_i or arsenate stimulates Ca^{2+} accumulation by mitochondria by preventing matrix alkalinization and elimination of membrane potential (33), had a much lower effect on lipid peroxidation.

Regarding the stimulation of lipid peroxidation by P_i, Fig. 3*A* demonstrates that mitochondrial swelling induced by 4 mM P_i (*line d*) can be significantly inhibited by sorbate (*line b*), a putative quencher of triplet carbonyl species (26). This inhibition is enhanced by the simultaneous presence of cyclosporin A (*line a*), but not of BHT (*line c*). This suggests that sorbate inhibits only the component of membrane permeabilization related to lipid peroxidation and not by MTP opening. Indeed, Fig. 3*B* shows that sorbate was not capable of inhibiting swelling induced by 1 mM P_i (*line a*).

In a previous report we have demonstrated that reduced coenzyme Q is the site for electron leakage responsible for Ca^{2+}-induced ROS production in liver mitochondria (20). In order to ascertain the proposition that mitochondrial damage induced by Ca^{2+} plus P_i is mediated by mitochondrial generated ROS at the level of coenzyme Q, we performed experiments with carbonyl cyanide *p*-trifluorophenylhydrazone-deenerziged mitochondria treated with antimycin A in the presence or absence of succinate, which maintains coenzyme Q in the reduced or oxidized form, respectively (18). Fig. 4 shows that in the absence of succinate, a condition under which H_2O_2 production is minimum (20), 4 mM P_i addition did not cause mitochondrial swelling (*line a*), while in the presence of succinate,

TABLE I
TBARS formation by mitochondrial preparations in the presence of Ca^{2+} plus P_i or arsenate

The experimental conditions were similar to those of Fig. 1.

Conditions	TBARS formation
A. Effect of P_i concentration, arsenate, or acetate (in presence of 10 μM Ca^{2+})	
	nmol TBARS/mg protein/10 min
No additions	0.30 ± 0.01[a]
1 mM EGTA	0.16 ± 0.04
1 mM P_i	0.93 ± 0.03
2 mM P_i	1.91 ± 0.01
3 mM P_i	2.24 ± 0.05
4 mM P_i	2.48 ± 0.07
5 mM P_i	2.67 ± 0.02
2.5 mM arsenate	2.59 ± 0.05
20 mM acetate	0.91 ± 0.03
B. Effect of EGTA, catalase, BHT, or sorbate on 4 mM P_i plus 10 μM Ca^{2+} induced TBARS formation	
	nmol TBARS/mg protein/10 min
1 mM EGTA	1.10 ± 0.03
2 μM catalase	1.14 ± 0.05
5 μM BHT	1.12 ± 0.03
100 μM sorbate	1.15 ± 0.01

[a] Values represent averages of six different experiments ± S.D.

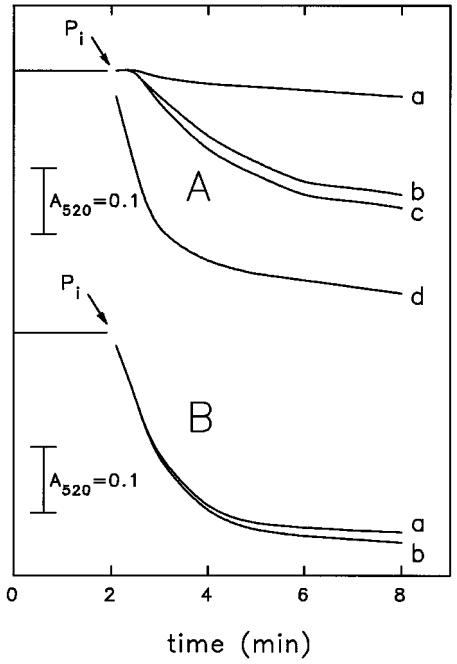


FIG. 3. **Effect of sorbate on mitochondrial swelling induced by P_i and Ca^{2+}.** RLM (0.4 mg/ml) were added to standard reaction medium, containing the following: *panel A*: *a*, 100 μM sorbate and 1 μM cyclosporin A; *b*, 100 μM sorbate; *c*, 100 μM sorbate and 5 μM BHT; *d*, no additions; *panel B*: *a*, 100 μM sorbate; *b*, no additions. P_i (4 mM in *panel A*, 1 mM in *panel B*) was added after 2 min of mitochondrial preincubation.

which increases electron leakage at the level of coenzyme Q (18, 20), a fast and extensive swelling was observed (*line d*). This swelling was partially inhibited by 100 μM sorbate (*line b*) or 5 μM BHT (*line c*) indicating that there is a component of membrane permeabilization related to lipid peroxidation. In addition, Fig. 5 shows that additions of arsenate (*line a*) or P_i (*lines b* and *c*), but not acetate (*line d*), greatly stimulate the mitochondrial production of H_2O_2 caused by Ca^{2+} alone (*line e*).

The nature of the synergism between P_i and Ca^{2+} in the process of lipid peroxidation was assessed by using a model system consisting of PC/DCP liposomes and the well known

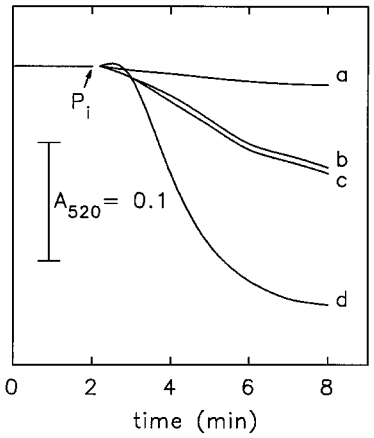


FIG. 4. **Effect of coenzyme Q redox state on mitochondrial swelling induced by Ca^{2+} and P_i.** RLM (0.4 mg/ml) were incubated in reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Hepes buffer, pH 7.2, 5.0 μM rotenone, 2 μM antimycin A, 1 μM carbonyl cyanide *p*-trifluorophenylhydrazone, and 300 μM Ca^{2+}) containing: *a*, no additions; *b*, 2 mM succinate plus 100 μM sorbate; *c*, 2 mM succinate plus 5 μM BHT; *d*, 2 mM succinate. P_i (4 mM) was added where indicated.

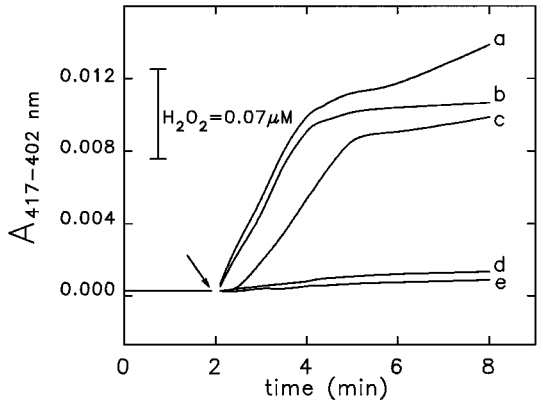
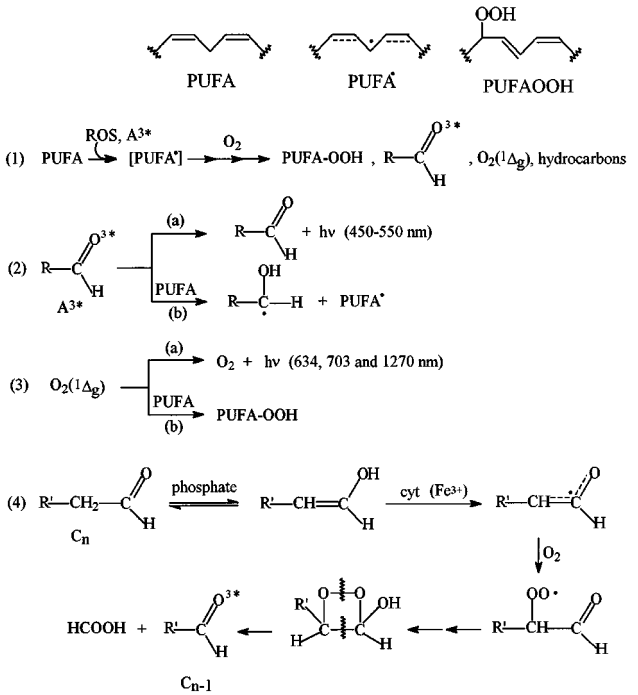


FIG. 5. **Production of H_2O_2 induced by P_i, arsenate, or acetate additions to Ca^{2+}-loaded mitochondria.** RLM (0.3 mg/ml) were added to standard reaction medium containing 1 μM horseradish peroxidase, 1 μM cyclosporin A, and 5 μM BHT. Where indicated by the *arrow*, 2.5 mM arsenate (*a*), 4 mM P_i (*b*), 1 mM P_i (*c*), or 20 mM acetate (*d*) was added. *Line e* represents a control experiment with no additions.

triplet acetone-generating system HRP/IBAL (26); DCP confers negative charge to the liposomes. It is well established that triplet acetone can abstract an allylic electron from linolenic and arachidonic acid (see Scheme II, Reaction 2b) (25), initiating their peroxidation. We hypothesized that: (i) whichever the mechanism, the interaction of P_i with the membrane lipid phase would be facilitated by neutralization of the membrane surface negative charge by Ca^{2+}; and (ii) the phosphate property to catalyze the enolization of aldehyde lipid peroxidation products (25) would favor their further α-peroxidation yielding extra triplet carbonyls (Reaction 4), thus increasing the rate of polyunsaturated fatty acid peroxidation (Reaction 2b). Indeed, Fig. 6 shows that P_i stimulated lipid peroxidation (as judged by TBARS absorbance increase at 535 nm) in this model system, in a dose-dependent fashion, when a constant Ca^{2+} concentration (0.5 mM) was present (*squares*). It should be mentioned that in the absence of Ca^{2+} the rate of TBARS production (during 10 min) was 50% smaller at a P_i concentration of 5 mM (not shown). This supports the hypothesis that the P_i effect is potentiated by the electrostatic interaction between Ca^{2+} and DCP. This is well documented by Fig. 6 (*circles*) showing that this physical effect of Ca^{2+}, under constant P_i concentration, increases by increasing its concentration from zero to 1 mM. In



SCHEME II. **Chain length amplification of polyunsaturated fatty acid peroxidation by triplet carbonyl products.** Peroxidation of PUFA is initiated by either HO· radicals or triplet carbonyls (A^3*) to give hydroperoxides (PUFA-OOH) and fragmentation products, which include triplet aldehydes, singlet oxygen and short hydrocarbons (Reaction 1). The chain propagation of PUFA peroxidation can thus be amplified (Reaction 2b) by A^3*. The typical chemiluminescence which accompanies the process is attributed to radiative deactivation of the triplet products (Reaction 2a) and singlet oxygen (Reaction 3a). Singlet oxygen can also undergo 1,3-cycloaddition to PUFA to yield PUFA-OOH (Reaction 3b). In the presence of phosphate and cytochrome *c* (*cyt* Fe^{3+}), the peroxidation-generated aldehydes can behave similarly to the isobutanal/HRP system (Scheme I) producing lower aldehydes homologous in the triplet state (Reaction 4), which are able to reinitiate the peroxidation cycle (Reaction 1).

contrast, when phosphate (5 mM) was replaced by Tris at the same concentration, TBARS production was negligible and Ca^{2+} had no effect (data not shown). The saturation kinetics displayed by both experiments in Fig. 6 may be rationalized in terms of the liposome concentration being limitant to the peroxidative process.

DISCUSSION

Previous results (17–19) from our laboratory show that mitochondrial membrane permeabilization induced by Ca^{2+}, under conditions of oxidative stress associated with *t*-butyl hydroperoxide reduction, is mediated by the oxidative attack of reactive oxygen species to membrane protein thiols. This causes thiol cross-linkage and high molecular weight protein aggregate production that may open a membrane pore upon the binding of Ca^{2+} (34). These alterations of the inner mitochondrial membrane seem to be related to the state designated as mitochondrial membrane permeability transition, characterized by the reversible opening of a membrane protein pore. The current understanding is that MTP is a protein channel (for review, see Refs. 9 and 10) that is opened by low membrane potential (32, 35), thiol cross-linking agents (34, 36), and various inducing agents in the presence of Ca^{2+} and closed by EGTA, ADP, Mg^{2+} (37), sulfydryl reducing agents (21, 36), protonation of the mitochondrial matrix (32), and submicromolar concentrations of cyclosporin A (30).

This work provides evidence that in the presence of P_i the increase in membrane permeability caused by Ca^{2+} can be mediated by alterations of both proteins and lipids. The rela-

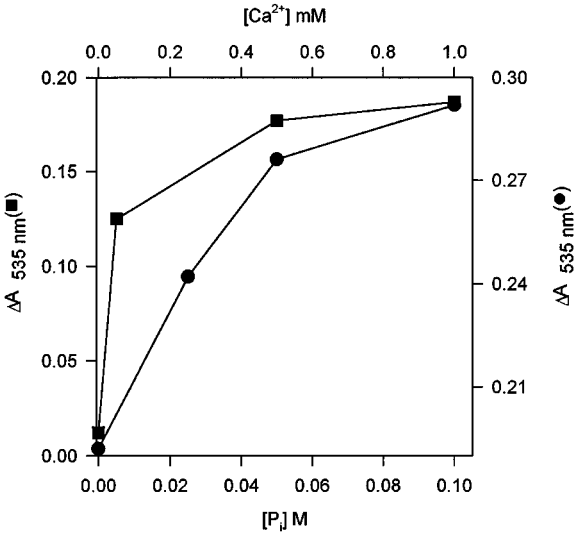


FIG. 6. **Enhancement of lipid peroxidation by P_i and Ca^{2+} in the model system consisting of PC/DCP 20 mol% liposomes.** Lipid peroxidation was initiated by the triplet acetone-generating system HRP (5 μM)/IBAL (1 mM). PC/DCP liposomes typically contained [PC + DCP] ~ 3.5 mM. The vesicles were incubated with the HRP/IBAL system at 37 °C during 20 min, and then samples were taken for TBARS determination, as described under "Materials and Methods." *Squares* represent the effect of P_i concentration in the presence of 0.5 mM Ca^{2+}. *Circles* represent the effect of Ca^{2+} concentration in the presence of 5 mM P_i.

tive importance of these processes depends on P_i concentration. At high P_i concentrations, both lipid peroxidation and MTP opening occur. This is illustrated in Fig. 1*A* by the partial protection conferred either by cyclosporin A or BHT and the total protection obtained when both compounds were present simultaneously. However, at lower P_i concentrations, opening of the MTP appears to be the main alteration, since cyclosporin A alone provides an almost complete mitochondrial protection (Fig. 1*B*).

The role of P_i seems to be mediated by a stimulation of mitochondrial ROS production at the level of reduced coenzyme Q, since maintenance of this coenzyme in an oxidized state prevents H_2O_2 production and mitochondrial swelling (see Fig. 4). Indeed, Kowaltowski *et al.*[2] provided evidence that, as in the case of *t*-butyl hydroperoxide plus Ca^{2+} (19), P_i plus Ca^{2+} did not cause membrane permeabilization when mitochondria were incubated in the absence of O_2. The mechanism by which P_i increases H_2O_2 production is still unknown but requires the presence of matrix Ca^{2+}. The experiments carried out with liposomes (Fig. 6) support the notion that Ca^{2+} binding to inner membrane surface (cardiolipins) decreases negative surface charge density (38) easing the access of P_i to the membrane lipid phase where it may stimulate lipid peroxidation (see Scheme II). This may render the respiratory chain more susceptible to electron leakage and ROS production.

With reference to the mechanisms by which P_i or arsenate stimulate lipid peroxidation, it is important to recall their property to catalyze tautomerization of aldehydes (C_n) (25), a process that may be followed by cytochrome-catalyzed peroxidation of the enols formed (Ref. 39 and Scheme II, Reaction 4). This leads to increased production of the C_{n-1} aldehyde in the triplet state (triplet carbonyls), which stimulates lipid peroxidation (Scheme II, Reaction 2b). Lipid peroxidation is a chemiluminescent process, due to the production of triplet carbonyls and singlet oxygen (Scheme II, Reactions 2a and 3a) (40–42). Triplet carbonyls (Scheme II, Reaction 2b) can initiate the peroxidation of polyunsaturated fatty acids (PUFA) (25) and singlet oxygen (Scheme II, Reaction 3b) directly produces the final product (PUFA-OOH) by 1,3-cycloaddition (42). This would amplify the propagation length of the peroxidative reaction chain. The stimulation by P_i of triplet carbonyl formation is strongly supported by the observed inhibitory effect of sorbate, a triplet carbonyl quencher, on both TBARS yield and mitochondrial swelling. Sorbate has been shown to quench triplet acetone formed by the IBAL/HPR system at the micromolar range (26). Regarding the participation of cytochromes in this process, recent results from our laboratory (39) reveal that mitochondria have the ability to promote a cytochrome-mediated P_i-stimulated oxidation of diphenylacetaldehyde. This oxidation produces triplet benzophenone and is followed by a BHT-sensitive process of membrane permeabilization.

In addition, the data presented in this paper is relevant regarding the discrimination of factors determinating reversibility *versus* irreversibility of Ca^{2+}-induced cell injury under conditions of ischemia and reperfusion. Although membrane permeabilization determined by MTP opening, in studies *in vitro*, is easily reversed by pore closure induced by EGTA, disulfide reductants, cyclosporin A, adenine nucleotides, etc. (9) under *in vivo* conditions, in which phosphate concentrations attain high levels, overlapping of the peroxidative process seems to be an important event leading to irreversible or lethal cell injury.

Acknowledgments—We thank Luis H. G. Ribeiro and Matheus P. C. Vercesi for excellent technical assistance.

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Membranes and Bioenergetics:

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J. Biol. Chem. 1996, 271:2929-2934.
doi: 10.1074/jbc.271.6.2929