Ascorbic Acid Is an Endogenous Cytosolic Inhibitor of ATP-supported Rat Liver Mitochondrial Calcium Transport*

Harry LeVine, III, Duane D. Bronson, Roger Khouri, and Naji E. Sahyoun

From the Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

ATP-supported but not Site I or Site II respiratory chain-linked $^{46}$Ca$^{2+}$ transport into isolated rat liver mitochondria is profoundly inhibited by a small molecule present in the cytosolic fraction. This inhibitor was found to be identical with ascorbic acid in a number of chemical properties, cytosolic abundance, susceptibility to ascorbate oxidase, and to agents that otherwise block the effect of authentic ascorbic acid. Experiments with a variety of free radical scavengers and glutathione indicated that ascorbate inhibition of calcium transport is mediated through a 1-electron-free radical mechanism rather than a conventional 2-electron reaction. Calcium transport mechanisms may, therefore, be a target in the pathophysiology of disease processes that influence the intracellular ratios and levels of ascorbate and physiological radical scavengers.

Mitochondrial Ca$^{2+}$ transport and storage have been the subject of considerable experimentation which has been reviewed extensively (1-7). Independent influx and efflux pathways have been clearly identified. Isolated mitochondria take up Ca$^{2+}$ via an electrochemical, potential-dependent uniporter which can be energized either by an oxidizable substrate such as succinate or by ATP, accompanied by proton extrusion (1, 3, 5, 7-9). Ca$^{2+}$ uptake and retention are facilitated by anions such as phosphate, bicarbonate or organic weak acid anions (5, 10). Intramitochondrial Ca$^{2+}$ is partially buffered by phosphate, and the intramitochondrial formation of calcium phosphates releases H$^+$, serving as a proton buffer as well (3, 11). While it is thermodynamically possible to reverse the Ca$^{2+}$ uptake uniporter, this reaction has little physiologic significance (12, 13). Ca$^{2+}$ efflux from mitochondria actually occurs via an H$^+/Ca^{2+}$ antiporter (3, 6, 14, 15) which usually operates at a lower rate than the uptake uniporter. These independent uptake and efflux pathways permit mitochondrial Ca$^{2+}$ recycling, achieving an extramitochondrial steady state set level of free Ca$^{2+}$ which is as low as $3 \times 10^{-7}$ M (13, 16-19). The fact that mitochondrial poisons seem to exert significant effects on cytosolic Ca$^{2+}$ levels and on the ability of cells to handle a Ca$^{2+}$ load (1, 3) has also been considered as supportive evidence for the physiologic importance of mitochondrial Ca$^{2+}$ fluxes.

Several physiological factors have been identified which modulate mitochondrial Ca$^{2+}$ fluxes. These include other ions such as Mg$^{2+}$ which inhibits Ca$^{2+}$ uptake (6) and Na$^+$ which stimulates Ca$^{2+}$ release in heart and brain mitochondria (6, 13, 20, 21). The release of oxidized pyridine nucleotides seems to be particularly important in regulating Ca$^{2+}$ fluxes leading to a reduction in Ca$^{2+}$ uptake when the intracellular level of ATP is diminished (22, 23). Cyclic AMP (24), saturated and unsaturated fatty acids (25-27), thiols (28), and polyamines (16) have been also implicated in regulating mitochondrial Ca$^{2+}$ transport.

The present study was designed to detect the possible occurrence of and to probe the mechanism of action of cytosolic components which regulate mitochondrial Ca$^{2+}$ uptake. Here we describe and characterize an endogenous inhibitor of mitochondrial Ca$^{2+}$ uptake. Evidence is provided that this inhibitor is identical with ascorbate and that its mechanism of action involves free radicals rather than the more conventional 2-electron redox reactions. These findings may imply ascorbate-sensitive (free radical) mechanisms in the physiologic and pathophysiologic regulation of intracellular Ca$^{2+}$ metabolism.

EXPERIMENTAL PROCEDURES

Materials—$^{46}$CaCl$_2$ (32 mCi/mg) and dehydroascorbate were purchased from ICN. 0.45-μm HAWP filters were obtained from Millipore. L-Ascorbic acid was an Eastman Kodak product while D-arabinoascorbic acid was supplied by Fisher. Ruthenium red and the Ca$^{2+}$ ionophore A23187 were from Sigma. Ascorbate oxidase purified from green zucchini squash was kindly provided by Dr. E. J. Diliberto, Jr., Department of Medicinal Biochemistry, Wellcome Research Laboratories.

Methods—Liver mitochondria from male Sprague-Dawley rats (130-180 g) were prepared by differential centrifugation in sucrosemannitol buffers without EDTA (29) and were finally suspended in 50 mM Tris-Cl, pH 7.4, containing 0.25 M sucrose. $^{46}$Ca$^{2+}$ uptake was measured in 1-ml aliquots of a buffer containing 15 mM Tris-Cl, pH 7.4, 120 mM KCl, 20 mM NaCl, 50 μM CaCl$_2$, 1 μCi of $^{46}$Ca$^{2+}$ (20 mCi/mmol), 5 mM MgCl$_2$, 0.8 M sodium phosphate, 2.4 mM ATP and 10 μg/ml of mitochondrial protein, unless otherwise specified. The reaction mixture was prewarmed at 37 °C for 5 min and the uptake reaction was initiated by adding 100 μl of the appropriate ice-cold mitochondrial suspension. The reaction was terminated after 3 min, unless indicated otherwise, by vacuum filtration through 0.45-μm Millipore HAWP filters followed by washing the filters with 3 ml of 50 mM Tris-Cl, 0.25 M sucrose, pH 7.4. Similar results could also be obtained after a 20-min incubation or in a nonionic assay mixture containing isomolar sucrose. Correction for adsorption of calcium was made by the addition of the calcium ionophore, A23187, to control assays. All (>95%) of the ionophore-releasable calcium uptake was determined to be of mitochondrial origin by its sensitivity to 10 μM ruthenium red. Cytosol from liver was prepared by Poltronon homogenization in 3 volumes of 50 mM Tris-Cl, pH 7.6, containing 0.25 M sucrose at 4 °C. The homogenate was centrifuged consecutively at 40,000 × g for 15 min and then at 300,000 × g for 90 min. The final supernatant was used as a cytosolic preparation. Protein concentration was determined by the Coomassie blue R-250 method (30) after comparison to the Lowry method (31). All ascorbate solutions were freshly dissolved and the pH adjusted just prior to use.

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RESULTS

The initial mitochondrial Ca$^{2+}$ uptake system contained ATP, succinate, P, Mg$^{2+}$, KCl, NaCl and Ca$^{2+}$. The presence of respiratory substrates in addition to ATP and P, failed to promote further Ca$^{2+}$ accumulation by the mitochondria. Results were similar with ATP in the presence or absence of pyruvate and malate (Site I substrates), $\beta$-hydroxybutyrate and succinate (Site II substrates), or a mixture of the four compounds (Table I), indicating that ATP was the dominant energy source for the Ca$^{2+}$ uptake process. Such results are consistent with the reported ATP requirement for the ability of mitochondria to maintain a Ca$^{2+}$ load and for steady state Ca$^{2+}$ recycling (11, 13, 32). Thus, in subsequent experiments ATP was employed as the energy source for Ca$^{2+}$ uptake.

Identification of Endogenous Inhibitor As Ascorbic Acid—
Cytosol fractionated by gel permeation chromatography on Sephadex G-25 alters ATP-supported Ca$^{2+}$ transport (Fig. 1). The void volume of the column contains a mixture of stimulatory and inhibitory substances and is the subject of continuing investigation. An inhibitory activity elutes slightly before the included column volume. Previous studies in our laboratory (33) had indicated that ascorbic acid, but not its oxidized form, dehydroascorbic acid, would block ATP-supported Ca$^{2+}$ uptake by mitochondria in contrast to its utilization at Site I for electron transport-supported Ca$^{2+}$ uptake in the presence of an appropriate electron acceptor (8, 22). This vitamin is present at significant concentrations in acid extracts of tissues (2-3 $\mu$g/g, wet weight) (34) and would, therefore, be predicted to be found in high concentration in liver cytosol. The peak fraction of inhibitory activity was subjected to a variety of tests to determine its effect on the bioactivity.

Chemical properties including charcoal adsorbability, acid and base stability and base lability were consistent with the presence of ascorbate, while free fatty acids, also potent inhibitors of Ca$^{2+}$ uptake (35) were ruled out by lack of extraction of inhibitory activity by organic solvents at pH 4. Furthermore, treatment of the peak fraction of inhibitory activity with ascorbate oxidase destroyed the inhibitory activity (Table II). Determination of ascorbate concentration by the ferrozine method (36) indicated sufficient levels of the vitamin to cause the observed inhibition (Fig. 1), as determined from comparison with authentic L-ascorbic acid. Treatment of unfractionated cytosol with ascorbate oxidase eliminated the inhibitory component present therein. More than 90% of the endogenous ascorbate measurable by ferrozine in the cytosol was destroyed by the treatment.

Further fractionation of the inhibitory activity by high pressure liquid chromatography on a Partisil 10 SAX anion exchange column (37) yielded activity that co-migrated with authentic ascorbate and displayed an $A_{245nm}$ commensurate with the amount of inhibitory activity and amount of ascorbate measured by ferrozine. This activity was destroyed by treatment with ascorbate oxidase or alkali.

Taken together, these data confirmed the presence of ascorbate in liver cytosol in a form capable of modulating Ca$^{2+}$ transport activity.

Characterization of Ascorbate Effects on Mitochondrial Ca$^{2+}$ Fluxes—The effects of ascorbate on mitochondrial Ca$^{2+}$ transport in the presence of different energy sources were assessed and are recorded in Table I. Inhibition of transport by ascorbate occurred only when ATP was present. Ascorbate was ineffective against transport supported by succinate or $\beta$-hydroxybutyrate in the absence of ATP. Site I and Site II substrates, in the presence of ATP, failed to alter the inhibition observed with ascorbate with ATP as the sole substrate. Thus, although energy derived either from hydrolysis of ATP

![Fig. 1. Ascorbate content of the endogenous inhibitor of Ca$^{2+}$ uptake isolated by Sephadex G-25 gel permeation chromatography. 0.8 ml of cytosol prepared as described under "Experimental Procedures."](http://www.jbc.org/)

\[
\begin{array}{c|c|c|c}
\text{Substrate} & \text{Ca$^{2+}$ uptake} & \% \text{Inhibition by ascorbate} \\
\hline
\text{Buffer} & 172 & 37 & 79 \\
\text{ATP} & 138 & 32 & 77 \\
\text{ATP + malate + pyruvate} & 212 & 81 & 62 \\
\text{ATP + succinate + $\beta$-hydroxybutyrate} & 209 & 34 & 84 \\
\text{ATP + malate + pyruvate + succinate + $\beta$-hydroxybutyrate} & 100 & 125 & 50 \\
\text{Succinate + $\beta$-hydroxybutyrate + 10 mM NaF} & 671 & 39 & 94 \\
\text{ATP + 10 mM NaF} & 46 & 4L & 128 \\
\end{array}
\]

**Table I**

Dominance of ATP in ATP/respiratory substrate mixtures and the effect of added ascorbate

Mitochondrial Ca$^{2+}$ uptake was measured after 3 min at 37 °C as described under "Experimental Procedures." ATP was present at 2.4 mM in the assay, while the other substrates were each added to a final concentration of 1 mM. The concentration of ATP used was slightly less than the apparent $K_m$ for the nucleotide. (−) indicates stimulation.
or from oxidation of respiratory substrates may be utilized to
drive the electrophoretic uptake of Ca^{2+} (8, 38), some aspect
of the dominant ATP-driven system is differentially affected
by ascorbate.

The kinetics of Ca^{2+} uptake by mitochondria under the
assay conditions described are presented in Figs. 2-4. The
main effect of ascorbate appears to be the inhibition of Ca^{2+}
uptake (Fig. 2). Ascorbate failed to cause Ca^{2+} efflux from
mitochondria treated with ruthenium red (Fig. 2, right). How-
ever, a brief and transient stimulation of mitochondrial Ca^{2+}
efflux may account for the biphasic time course observed at
early time points (Fig. 2, left). All ascorbate effects are com-
plete by 2 min, so uptake measurements were routinely made
at 3 min to afford as early a time point as possible subsequent
to full expression of the inhibition. No further differences
were noted in Ca^{2+} retention up to 20 min. Effects of ascorbate
were reversible by washing of the mitochondria. Increasing
concentrations of Ca^{2+} appeared to sensitize ATP-supported
Ca^{2+} uptake to ascorbate, lowering the IC_{50} dramatically for a
given amount of mitochondrial protein (Fig. 3). Fig. 4 (top,
left and right) characterizes the ATP and Mg^{2+} requirements
of mitochondrial Ca^{2+} transport in the absence of added
respiratory substrates. The inhibition of uptake caused by
ascorbate is an effect on the V_{max} of transport with little
change of apparent \( K_{m} \) for ATP. Decreases of about a factor
of 2 were noted for V_{max} and \( K_{m} \) for Mg^{2+}. The amount
of ascorbate required to inhibit uptake is strongly dependent
upon the mitochondrial protein concentration, requiring
higher dosages of the vitamin to inhibit uptake into more
mitochondria (Fig. 4). This may be due in part to the meta-
bolic activity of mitochondria inactivating the ascorbate (see
next section). The ascorbate effect seemed to be constant
within the pH range 6.9–8.0 and was not stereospecific. No
effect of ascorbate on ATP transport (39) or membrane po-
tential (40) in the presence of ATP was detectable. Phosphate
ion co-transport was not limiting in the presence of ascorbate.
Table III shows that high phosphate concentrations actually
potentiate the ascorbate inhibition.

Involvement of Free Radicals in the Ascorbate Effect—A
redox substrate often used in the place of ascorbate (\( E_{H^{+}}^0 =
+0.689 \) V) in experiments involving mitochondrial respira-
tion, phenozine methosulfate (\( E_{H^{+}}^0 = +0.058 \) V), failed to
inhibit ATP-supported Ca^{2+} transport at up to 10 \( \mu \)M under
conditions where 0.3 \( \mu \)M ascorbate produced full inhibition.
This suggested that the effect of ascorbate was not mediated
by its ability to participate in redox reactions involving the
electron transport chain. An explanation of ascorbate effects
based on generation of \( \mathrm{H}_{2}\mathrm{O}_{2} \) is unlikely since the addition of

![FIG. 2. Time course of mitochondrial Ca^{2+} uptake. Left, time
course of ATP-supported Ca^{2+} uptake into mitochondria in the
presence and absence of ascorbate. Ten micrograms of mitochondrial
protein were added to 1 ml of prewarmed assay mix at \( t = 0 \). At the
indicated times uptake was terminated by filtration. O, refer to uptake
in the absence of ascorbate; \( \bullet \), refer to uptake in the presence of 100
\( \mu \)M ascorbate. \( \Delta \), indicates points obtained with 100 \( \mu \)g/ml of mito-
ochondria incubated on ice with 1 \( \mu \)M ascorbate for 10 min before
aliquots were added to prewarmed reaction mixture to give the same
final mitochondrial and ascorbate concentrations. Right, time course of
Ca^{2+} retention after addition of ascorbate. Assay performed as
described for the left panel of this figure. At \( t = 3 \) min either buffer
(O), 10 \( \mu \)M ascorbate (\( \bullet \)), 10 \( \mu \)M ruthenium red followed immediately
by buffer (\( \Delta \)), or 10 \( \mu \)M ruthenium red followed immediately by 10
\( \mu \)M ascorbate (\( \Delta \)) were added to reaction mixtures containing 10 \( \mu \)g
of mitochondria. At appropriate times after these additions, uptake
was terminated by filtration.

![FIG. 3. Dependence of uptake on Ca^{2+} concentration. Left,
calcium concentration dependence of calcium uptake. Uptake was
determined after 3 min at 37 °C in the standard reaction mixture at
different Ca^{2+} concentrations. Right, increase in effectiveness of as-
orbate with higher Ca^{2+} concentration. Uptake was determined as
in the left panel to this figure in the presence of a range of ascorbate
concentrations at each of three total Ca^{2+} concentrations; 10 \( \mu \)M Ca^{2+}
(\( \bullet \)), 50 \( \mu \)M Ca^{2+} (\( \Delta \)), and 200 \( \mu \)M Ca^{2+} (\( \bullet \)).

![FIG. 4. Effect of ascorbate on Ca^{2+} uptake parameters. Top
left, effect of ascorbate on ATP concentration dependence of Ca^{2+}
uptake. The ATP concentration in the standard assay was varied in
the presence of control buffer (\( \bullet \), 0.1 \( \mu \)M ascorbate (O), or 3 \( \mu \)M
ascorbate (\( \Delta \)). Top right, effect of ascorbate on Mg^{2+} concentration
dependence of Ca^{2+} uptake. The MgCl\(_2\) concentration in the standard
assay was varied in the presence of control buffer (\( \bullet \), 0.8 \( \mu \)M
ascorbate (O), or 20 \( \mu \)M ascorbate (\( \Delta \)). Bottom left, Ca^{2+}
accumulation in the assay as a function of mitochondrial protein concen-
tration. Total uptake was less than 30% of added Ca^{2+} at the highest protein
concentration. Bottom right, effectiveness of ascorbate in the presence of
increasing mitochondria concentration. Ca^{2+} uptake was measured
under standard assay conditions with a range of ascorbate concen-
trations using 8 \( \mu \)g of mitochondrial protein (\( \bullet \)), 17 \( \mu \)g of mitochondrial
protein (O), or 86 \( \mu \)g of mitochondrial protein (\( \Delta \)).]
**TABLE III**  
Effect of phosphate on ascorbate inhibition of calcium transport

<table>
<thead>
<tr>
<th>Phosphate concentration (mM)</th>
<th>Ca²⁺ uptake with no ascorbate (ng ions Ca²⁺/mg protein)</th>
<th>Ca²⁺ uptake with 1 mM ascorbate (ng ions Ca²⁺/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>140</td>
<td>94</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>181</td>
<td>95</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>61</td>
<td>66</td>
</tr>
</tbody>
</table>

**TABLE IV**  
Effect of radical scavengers on ascorbate inhibition of calcium transport

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Concentration (10⁻³ M)</th>
<th>Type</th>
<th>% Reversal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethyldithiocarbamate</td>
<td>General</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Butylhydroxytoluene</td>
<td>General</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pyrocatechol</td>
<td>General</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>General</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1,2-Dimethoxyethane</td>
<td>OH⁻</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>1.4 x 10⁻¹ (1% v/v)</td>
<td>OH⁻</td>
<td>0</td>
</tr>
<tr>
<td>2,5-Dimethylfuran</td>
<td>10⁻⁴</td>
<td>O⁻</td>
<td>0</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>1.6 x 10⁻⁸</td>
<td>O₂⁻</td>
<td>0</td>
</tr>
</tbody>
</table>

Ascorbate inhibits ATP-driven mitochondrial Ca²⁺ uptake

Sodium phosphate (pH 7.4) was added at the indicated final concentrations in the presence or absence of ascorbate and Ca²⁺ uptake determined for 3 min at 37 °C as described under “Experimental Procedures.” The sensitivity of the mitochondrial Ca²⁺ transport system to ascorbate may account for the presence of the highest cellular specific activity of semidehydroascorbate reductase, a NADH-linked enzyme on the external face of the mitochondrial outer membranes (43). Reduction of the free radical would protect the Ca²⁺ transport system as well as regenerate the important cofactor ascorbate. In fact, the presence of 1 mM NADH in the assay completely blocked the inhibition of Ca²⁺ transport by 1 mM ascorbate.

**DISCUSSION**

This communication provides evidence for a relationship among three areas of biochemical research: the mechanism of action of ascorbate, free radical biochemistry and intracellular Ca²⁺ metabolism. It appears that ascorbate inhibits ATP-supported mitochondrial Ca²⁺ uptake via a mechanism involving free radicals. This inhibition is subject to further regulation by the ubiquitous intracellular component, GSH.

Ascorbate is sequestered by cells achieving concentrations up to 2-5 μmol/g (34). This vitamin is active in promoting a number of important biological responses such as chemotaxis (44), lymphocyte function in the immune response (45), secretion processes (46, 47), neurotransmitter release (48), brain catecholamine actions (49), platelet arachidonate metabolism (50), phagocytosis (51) and smooth muscle contractility (52). Ascorbate also appears to modulate cyclic nucleotide levels (53) and several aspects of tissue metabolism of Ca²⁺ (54, 55). Although the molecular locus of action of ascorbate in these processes has not been clearly identified, it may be of some relevance that the events affected involve the participation of the Ca²⁺ ion at some point.

Ascorbate present in unfractionated cytosol containing endogenous GSH is capable of inhibiting ATP-supported Ca²⁺ uptake into mitochondria, an inhibition abolished by treatment of the cytosol with ascorbate oxidase. The observation of ascorbate inhibition of Ca²⁺ transport in this crude system supports the physiological relevance of the inhibition. Furthermore, blockage of the effect of purified ascorbate by GSH concentrations found in cytosol suggest that the cytosol contains other entities that either may potentiate the ascorbate effect, or alternatively render GSH inaccessible, or both. Compartmentalization of these agents may also regulate the free cytosolic concentrations.

The mechanism by which ascorbate exerts its multiplicity of effects may be traceable to this incipient triketene’s rather exotic chemistry leading to oxidative and peroxidative reactions (56, 57), as indicated in Fig. 5.

The cell has evolved mechanisms to control the chemistry of some of these side reactions of free radicals such as the presence of high concentrations of reducing or radical quenching agents, in particular GSH, ergothionin, uric acid and other purines (34). The concept of a cellular system to buffer free radical reactions has been considered in diverse processes, including aging (58), differentiation and mitogenesis (59), carcinogenesis (60, 61), tissue damage due to ionizing radiation (62, 63), ischemic changes in myocardium (64) and brain...
Ascorbate Inhibits ATP-driven Mitochondrial Ca\textsuperscript{2+} Uptake

(65), as well as drug toxicity (66, 67). Such a buffer would also serve to protect the calcium regulatory systems of the cell in their maintenance of a low cytosolic Ca\textsuperscript{2+} concentration. Alterations in the cell's ability to deal with toxic metabolites such as extensive lipid peroxidation or production of such as Na\textsuperscript{+}, K\textsuperscript{+}, or phosphate is not limited to a treated with ascorbate and the membrane potential remains some component of the uptake uniporter coupled to the utilization of ascorbate in this intact mitochondria suggest that this could provide an additional tool for dissecting effects on cell function. The effects of ascorbate on Ca\textsuperscript{2+} transport in mitochondria suggests that this may serve to protect the calcium regulatory systems of the cell in influencing Ca\textsuperscript{2+} transport in mitochondria.

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