Stimulation of Mitochondrial Calcium Uptake by Light during Growth of Corn Shoots

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ABSTRACT

Ca2⁺ uptake in mitochondrial fractions, isolated on Percoll discontinuous density gradients, from light- and dark-grown corn (Zea mays L. var W64A X W182E) shoots was characterized by dual wavelength spectroscopy and the Ca²⁺-sensitive dye murexide. In light-grown seedlings, the rate of mitochondrial Ca²⁺ uptake was about 40 nanomoles per minute per milligram of mitochondrial protein. A portion of the Ca²⁺ uptake required an exogenous supply of ATP (65%) while the remaining 35% was the respiratory substrate-dependent reaction. Ruthenium red (2 micromolar) completely inhibited both ATP- and substrate-dependent reactions. There was no detectable Ca²⁺ efflux from the mitochondria with the inhibitor. When the mitochondrial fraction was prepared from the dark-grown shoots, the rate of uptake, in particular the ATP-dependent reaction, was greatly reduced. The dark treatment caused a reduction in mitochondrial Ca content which is largely due to the reduction of Ca associated with the mitochondrial membrane rather than to a reduction of Ca in the soluble matrix. Free Ca²⁺ ion has been considered to play a role regulating metabolic activities and some of its functions are mediated through a Ca²⁺-calmodulin system in plants (2–4, 10, 13, 17, 19). The cytoplasmic concentration of free Ca²⁺ is thought to be in the range of 10⁻⁶ to 10⁻³ M (1, 18) and this low Ca²⁺ concentration is maintained against high Ca²⁺ concentrations outside the cells and inside the various membrane systems. The regulation for maintaining the low cytoplasmic Ca²⁺ is thought to involve both the accumulation of Ca²⁺ in subcellular organelles, in particular mitochondria, through an active Ca²⁺ transport system (8, 17) and an active extrusion through the plasma membrane (4).

For the accumulation of Ca²⁺ by plant mitochondria, energy can be supplied by either substrate oxidation or exogenously added ATP (7, 8). Oligomycin specifically inhibits the ATP-driven Ca²⁺ uptake, but not the substrate-dependent uptake, suggesting that a high energy intermediate of oxidative phosphorylation is the direct energy source for mitochondrial Ca²⁺ uptake in plants (8) and in animals (9). Roux et al. (17) demonstrated that the active Ca²⁺ fluxes in purified mitochondria obtained from etiolated oat shoots are regulated by phytocrome. Their results indicate that the red light irradiation in vitro diminishes the net uptake rate, following the induction of Ca²⁺ efflux from mitochondria, and far-red light restores this rate to its dark control level when both succinate and ATP are used as the energy source. Dieter and Marmé (3) also suggested the regulation of mitochondrial Ca²⁺ fluxes by phytocrome; however, in their system the active accumulation of Ca²⁺ into the mitochondrial fraction from etiolated corn coleoptiles is inhibited when the seedlings are irradiated with far-red light. In any case, the properties of the active Ca²⁺ fluxes by plant mitochondria seem to be altered by light, through the action of phytocrome.

Most Ca²⁺ flux assays have been performed with mitochondria isolated from the etiolated plant tissues (4, 8, 17), because of the difficulty in isolating the preparation free from other organelles from the green tissues. However, the Percoll discontinuous density gradient method permits the isolation of pure and metabolically competent mitochondria from leaves (14). With this isolation method, alterations of mitochondrial Ca²⁺ fluxes and Ca accumulation can be followed during light/dark transitions. Variations in Ca²⁺ concentrations in both cytosol and mitochondria during light/dark transitions could be important in the regulation of Ca²⁺-requiring enzymes.

In this paper, we show that an ATP-dependent Ca²⁺ uptake by corn mitochondria is stimulated by growth in the light and that there is also an accumulation of Ca in the mitochondrial membrane under these conditions.

MATERIALS AND METHODS

Plant Materials. Corn seed (Zea mays L. var W64A X W182E) were planted in vermiculite and the seedlings were grown in a Toshiba Cold Chain growth chamber, model TGS-13L, with either a 12-h photoperiod for 5 d (light-grown corn) or 3 d followed by 2 d with no light (dark-grown corn), unless indicated. The primary leaves developed to about 3 cm in each case. Light (about 490 µE/m²·s) was supplied by Toshiba Yoko lamps DA400 (metal halide lamp) and fluorescent lamps, and the temperatures were maintained at 27°C. The plants were watered every morning with a nutrient medium containing 1 mM Ca²⁺ as described previously (12).

Isolation of Mitochondria by Density Gradient Centrifugation. All reagents and buffer solutions were prepared with double glass-distilled H₂O to minimize Ca²⁺ contamination, and all operations were carried out at 4°C. Whole shoots of 5 d seedlings were harvested, washed in water, dried with filter paper, and weighed. The shoots (40–60 g in fresh weight) were chopped with razor blades in extraction buffer consisting of 0.4 M mannitol, 0.1 M Hepes-KOH buffer (pH 7.5), 1 mM EDTA, 0.1% (w/v) BSA, and 0.6 (w/v) insoluble PVP, at a ratio of 1 g fresh weight/5 ml of buffer. The chopped materials were ground with mortar and pestle.
The mitochondrial fraction was isolated by a modification of the method described by Nishimura et al. (14). The homogenate was passed through four layers of gauze, centrifuged at 1,000g for 10 min to sediment debris, starch, chloroplasts, and aggregated organelles. The supernatant fraction was then centrifuged at 10,000g for 10 min to provide a crude mitochondrial pellet. The pellet was gently dispersed in 1 ml of suspending buffer containing 0.3 M mannitol, 20 mM Hepes-KOH (pH 7.5), and 0.1% (w/v) fatty acid free BSA. It was then layered on top of a discontinuous Percoll gradient. The discontinuous gradient comprised: A, 3 ml 60% (v/v) Percoll; B, 4 ml 45% (v/v) Percoll; C, 28% (v/v) Percoll; and D, 4 ml 5% (v/v) Percoll. In each case the Percoll solution contained 0.25 M sucrose, 20 mM Hepes-KOH buffer (pH 7.5), and 0.1% (w/v) fatty acid free BSA. Centrifugation was carried out at 30,000g for 30 min in a Beckman model L5-75 ultracentrifuge equipped with a SW 27-1 rotor. The mitochondrial fraction, which migrated to the interface between B and C, was carefully collected with a Pasteur pipet. To remove Percoll, the collected fraction was diluted 10 times with the suspending buffer and centrifuged at 10,000g for 15 min. The resulting loose mitochondrial pellet was again suspended in the buffer and centrifuged. The final mitochondrial pellet was gently dispersed in 0.5 ml of the same buffer.

Preparation of Submitochondrial Fractions. The purified mitochondrial pellet was shocked hypotonically using 2 ml of 20 mM Hepes-KOH buffer (pH 7.5). This suspension was then sonicated in a salt-ice mixture with a Branson sonifier, model 200, fitted with a microtip, operating at power setting 5 on a 50% duty cycle for 2 min. Following sonication, the suspension was centrifuged in the ultracentrifuge equipped with an SW50-1 rotor at 150,000g for 1 h. The pellet, resuspended in 2 ml of the same buffer, was designated the mitochondrial membrane fraction. The supernatant remaining above the pellet consisted of the soluble matrix of the mitochondria.

Measurement of O2 Consumption by Mitochondria. O2 uptake was followed polarographically at 30°C using a Clark-type electrode purchased from Hansatech Ltd. (Hardwick Industrial Estate, King’s Lynn, Norfolk, U.K.) as described by Nishimura et al. (14). The reaction medium contained 0.3 M mannitol, 10 mM Na-phosphate buffer (pH 7.2), 5 mM MgCl2, 10 mM KCI, 0.1% (w/v) fatty acid free BSA, and a known volume of the mitochondrial fraction in a total volume of 1 ml.

Assay of Ca2+ Uptake by Mitochondria. The uptake of Ca2+ was monitored by dual wavelength spectroscopy with murexide according to the method of Roux et al. (18) with some modifications. The reaction medium contained 0.3 M mannitol, 10 mM Hepes-KOH buffer (pH 7.1), 0.1% (w/v) fatty acid free BSA, 4 mM succinate, 0.5 mM NADH, 50 μM murexide, 2.5 mM MgCl2, 5 mM K2HPO4, 10 mM KCI, 0.2 mM CaCl2, and a known amount of mitochondrial fraction either in the presence or absence of 3 mM ATP, in a total volume of 0.8 ml. The uptake assay was initiated by the addition of the mitochondrial fraction and the change in A440–407 nm was monitored with a Hitachi dual-wavelength double beam spectrophotometer, model 356 for 10 min at 28°C. Both initial velocities and the actual accumulations of Ca2+ were measured. The time elapsed between the initial tissue extraction and the beginning of Ca2+-flux assays after the measurement of O2 uptake was less than 5 h.

Determination of ATP Pool Generated by the Mitochondria. The isolated mitochondria were incubated in the Ca2+ uptake reaction mixture, but treated with or without Ca2+ and/or respiratory substrates (succinate and NADH) for 10 min at 28°C in the absence of ATP. The reaction was terminated by adding 0.1 ml of 0.2 M HCl and the generated ATP was extracted from the mitochondria by disruption with freezing and thawing, twice. The reaction terminated at 0 time was used as the blank. After the disruption, the extract was neutralized by adding an equivalent amount of NaOH and the extract (0.5 ml) was mixed with 0.1 ml of 0.5 M Hepes-KOH buffer pH 7.5, containing 0.5 mM magnesium acetate and 0.4 ml of water. ATP was determined on 100 μl aliquots of the mixture, using Amino-Chem Glow photometer, and 100 μl of reconstituted luciferin-luciferase (Sigma, FLE-50).

Enzyme Assays and Analytical Methods. GDH2 activity was assayed in the NADH-dependent amination reaction (15) in the presence of 1 mM CaCl2. Succinate:Cyt c reductase activity was assayed in the presence or absence of 0.3 mM succrose as described by Douce et al. (5). The Ca content in the isolated mitochondrial fraction was determined with a Shimadzu atomic absorption/flame emission spectrophotometer, model AA646, equipped with a Graphite furnace atomizer, model GFA-3. Protein content was determined by the method of Lowry et al. (11).

RESULTS AND DISCUSSION

Integrity of the Corn Shoot Mitochondria. The intactness of the mitochondria purified by Percoll density gradient centrifugation was evaluated by measuring succinate:Cyt c reductase activity with and without 0.3 M succrose. This enzyme is located on the internal face of the inner mitochondrial membrane so that the integrity of the outer mitochondrial membrane is estimated by comparing the activity before and after the osmotic shock (5). The representative activity of the enzyme was obtained as follows: 242 and 19 nmol Cyt c reduced/min·mg of mitochondrial protein in the absence and presence of succrose from the light-grown corn and 318 and 41 for mitochondria from the dark-grown corn. Judging from these activities, 87 to 92% of the outer membrane of the isolated mitochondria was intact in each case.

The respiratory O2 uptake of the isolated mitochondrial fraction, using malate or succinate as substrates, is shown in Figure 1. The O2 electrode traces showed that the mitochondrial fraction obtained from both the light- and dark-grown corn shoots were able to oxidize these substrates and there was a good control by ADP, indicating preservation of energy-linked functions. Both mitochondrial fractions had high respiratory control ratios (about 5 for malate) and ADP-to-O ratios close to the predicted values. State 3 rates with malate and respiratory control ratio with either substrate in the mitochondrial fraction from the light-grown corn were slightly higher than that from the dark-grown corn. The preparations in each case were suitable for use in the Ca2+ uptake experiments.

Ca2+ Uptake by the Isolated Mitochondria from Light- and Dark-Grown Corn Shoots. According to the work by Hodges and Hanson (8), Ca2+ uptake rate by corn mitochondria is the highest when succinate is used as the respiratory substrate. Hence, we also used succinate when investigating the mitochondrial Ca2+ uptake from the light- and dark-grown corn. The mitochondrial fraction isolated from light-grown corn shoots showed typical saturation kinetics for the uptake of Ca2+ during a 10-min incubation time (Fig. 2A). The rate of Ca2+ uptake was linear when the initial velocity was plotted as a function of increasing mitochondrial fraction (Fig. 2B). However, total Ca2+ accumulation during 5 min incubation was not linear because of the saturation kinetic. About 65% of the total uptake was dependent on an exogenously supplied ATP and the rest (35%) was a respiratory substrate-dependent (succinate and NADH) reaction. As was seen with oat mitochondria (18), both ATP- and substrate-dependent reactions were completely inhibited by low concentrations of Ru red, which is known to inhibit the active Ca2+ uptake selectively without affecting respiratory activity and Ca2+ efflux from the mitochondria (18). Active extrusion of Ca2+.

2 Abbreviations: GDH, glutamate dehydrogenase; Ru red, ruthenium red.
from the corn mitochondria was not observed in the presence of Ru red.

The dark treatment reduced the rate of mitochondrial Ca\textsuperscript{2+} uptake (Table I). The ATP-dependent Ca\textsuperscript{2+} uptake was more sensitive than the substrate-dependent uptake. The method used in this assay is based on measuring changes in Ca\textsuperscript{2+} concentration outside the mitochondria so that a total balance between Ca\textsuperscript{2+} influx into and efflux from the mitochondria are measured. Thus, the reduced uptake could be due to either a reduced influx or a stimulated efflux. However, judging by the data with Ru red, the Ca\textsuperscript{2+} efflux was not contributing to the differences seen in mitochondria isolated from both types of corn shoots. It is, therefore, concluded that the mitochondria obtained from the dark-treated shoots have a reduced capacity for the uptake of Ca\textsuperscript{2+} in particular the ATP-dependent reaction.

In agreement with the reduced ability of the mitochondria to accumulate Ca\textsuperscript{2+}, the dark-grown corn shoots had lower total Ca content in their mitochondrial fraction (Table II). To demonstrate whether the dark treatment resulted in a reduction of Ca\textsuperscript{2+} in either the mitochondrial soluble fraction or in the mitochondrial membrane fraction, or both, the distribution of Ca was measured after submitochondrial fractionation. Cross contaminations were checked by measuring succinate:Cyt c reductase as the membrane marker (5) and NADH-GDH as the matrix marker (16). The actual distribution of Ca was corrected on the basis of these activities. With the dark treatment, the Ca content in the membrane fraction was only 58% of the value found in the light-grown corn. Ca in the soluble matrix from both types of corn was quite similar, indicating that the reduction in mitochondrial Ca content is due largely to the reduction in the membrane-associated Ca.

**Determination of ATP Pool Generated by Mitochondria Isolated from the Light- and Dark-Grown Corn Shoots.** According to the model of mitochondrial Ca\textsuperscript{2+} uptake in the literature (7, 8), a phosphorylated high energy intermediate in oxidative phosphorylation is thought to provide energy for the uptake. Since the substrate-dependent Ca\textsuperscript{2+} uptake by corn mitochondria was less affected by the light/dark treatment, the generation of the unidentified intermediate, which is also a phosphoryl donor of ATP formation (7, 8), appears to be similar in the two treatments. This prediction was tested by measuring mitochondrial ATP pool as the final product of oxidative phosphorylation after the incubation of mitochondria in the Ca\textsuperscript{2+} uptake assay mixture in the absence of ATP (Fig. 3). Mitochondrial fractions from light-
The mitochondrial Ca$^{2+}$ uptake was monitored by $A_{340-507}$ change with dual wavelength spectroscopy with 50 μm murexide. The rate of uptake was determined from the initial velocity and the Ca$^{2+}$ accumulation was obtained during 5 min incubation at 28°C. The reaction was started by addition of purified mitochondrial fraction from 5 d corn shoots with various light treatment: 5 d light (5L/0D), 4 d light and 1 d dark (4L/1D), and 3 d light then 2 d dark (3L/2D). The assay system contained 112, 90, and 85 μg of mitochondrial protein for the 5L/0D, 4L/1D, and 3L/2D corn, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca$^{2+}$ Uptake</th>
<th>Ca$^{2+}$ Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5L/0D</td>
<td>4L/1D</td>
</tr>
<tr>
<td>Complete</td>
<td>40.2</td>
<td>24.7</td>
</tr>
<tr>
<td>ATP (3 mM)</td>
<td>25.9</td>
<td>8.7</td>
</tr>
<tr>
<td>ATP (0 mM)</td>
<td>14.3</td>
<td>16.0</td>
</tr>
<tr>
<td>ATP + Ru red (2 μM)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*The ATP-dependent Ca$^{2+}$ uptake was calculated by subtracting the values with no ATP from the complete values.

Table II. Intramitochondrial Ca Distribution in Corn Shoots Grown under the Light or Darkness

The distribution of intramitochondrial Ca content was determined after the sonication of the mitochondrial fraction followed by ultracentrifugation at 150,000 g for 1 h. The Ca content in each submitochondrial fraction was calculated with correction for cross contamination of the different compartments by measuring the marker enzymes. For example, Ca in the matrix from the light-grown corn was calculated as follows: actual Ca level = 24.3 × 0.85 + 31.5 × 0.15, and this was divided by mg of mitochondrial protein contained. Protein content of the mitochondrial fraction was 381 and 315 μg for the light- and dark-treatment.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Marker Enzyme Activity</th>
<th>Ca Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated</td>
<td>Succinate:</td>
<td>NADH-GDH</td>
</tr>
<tr>
<td></td>
<td>Cyt c reductase</td>
<td>Measured</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corrected</td>
</tr>
<tr>
<td>% of sonicated fraction</td>
<td>nmol· fraction$^{-1}$</td>
<td>nmol· mg$^{-1}$· protein</td>
</tr>
<tr>
<td>Light Sonicated</td>
<td>100</td>
<td>56.9</td>
</tr>
<tr>
<td>Matrix</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>Membrane</td>
<td>80</td>
<td>15</td>
</tr>
<tr>
<td>Dark Sonicated</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Matrix</td>
<td>12</td>
<td>85</td>
</tr>
<tr>
<td>Membrane</td>
<td>88</td>
<td>15</td>
</tr>
</tbody>
</table>

and dark-grown corn contained 0.97 and 0.86 nmol ATP/mg protein, respectively, when the ATP pool was determined at 0 time incubation. Incubations without substrates showed little or no change in the ATP pool during 10 min regardless of the presence of Ca$^{2+}$. On the other hand, about 4.2 nmol ATP/mg protein was obtained as the pool when the mitochondrial fraction from either light- or dark-grown corn was incubated with substrates in the absence of Ca$^{2+}$. Addition of Ca$^{2+}$ to the substrate-containing assay system caused a slight reduction (8-13%) of the ATP pool with either mitochondrial preparation. Thus, the light treatment had no effect on the generation of ATP pool through the oxidation of substrates within the corn mitochondria. This could be explained by the fact that substrate-driven Ca$^{2+}$ uptake by mitochondria is similar in the light- and dark-treatments. The slight reduction in ATP pool size in the presence of Ca$^{2+}$ may reflect energy consumption due to the uptake of Ca$^{2+}$.

As described in this paper, the ATP-dependent Ca$^{2+}$ uptake system by mitochondria is thought to contribute the regulation of Ca$^{2+}$ content associated with mitochondrial membrane and possibly with the cytosolic Ca$^{2+}$ concentration in corn shoots during light/dark transitions. The mechanism for the light-stimulation of ATP-dependent Ca$^{2+}$ uptake is not yet known. If the phosphorylated high energy intermediate is the true energy source for Ca$^{2+}$ uptake, ATP should be converted to this intermediate by the reverse of ATP formation (8). The reaction may be activated by light. If mix-light irradiation used during the growth period here has the same function as a single beam irradiation of red light in vitro, our data do not agree with the observations by Roux et al. (17) where the red light diminishes the net Ca$^{2+}$ uptake, following stimulation of Ca$^{2+}$ efflux, by isolated oat mitochondria. Our results are similar to those reported by Dieter and Marme (3), but they did not incubate their mitochondrial fraction with ATP. Thus, it is difficult to develop a general picture of the light effect, because different plants, mitochondrial preparations, light treatments, and Ca$^{2+}$ uptake assay methods are used in each case. However, the action of phytochrome affected by the light treatment may be a possible explanation for development of the ATP-dependent Ca$^{2+}$ uptake system in corn mitochondria.

In pea chloroplasts, conversion of NAD to NADP is induced by light and the induction is known through the action of calmodulin-activated NAD kinase (10). Mitochondrial Ca$^{2+}$ up-
take has been reported to be either inhibited by calmodulin-antagonists (6) or unaffected by addition of calmodulin (2). We tested the effects of bovine brain calmodulin (5 μg) and a calmodulin antagonist, trifluoperazine (0.1 mm), on the mitochondrial Ca\(^{2+}\) uptake in the presence of ATP. With the mitochondria isolated from light- or dark-grown corn shoots, neither calmodulin nor trifluoperazine affected the rate of Ca\(^{2+}\) uptake (results not shown). Therefore, the involvement of calmodulin in the stimulation of ATP-dependent Ca\(^{2+}\) uptake system by light seems to be ruled out.

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LITERATURE CITED