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

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ABSTRACT

Ca\(^{2+}\) 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 Ca\(^{2+}\)-sensitive dye murexide. In light-grown seedlings, the rate of mitochondrial Ca\(^{2+}\) uptake was about 40 nanomoles per minute per milligram of mitochondrial protein. A portion of the Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) ion has been considered to play a role regulating metabolic activities and some of its functions are mediated through a Ca\(^{2+}\)-calmodulin system in plants (2–4, 10, 13, 17, 19). The cytoplasmic concentration of free Ca\(^{2+}\) is thought to be in the range of 10\(^{-7}\) to 10\(^{-3}\) M (1, 18) and this low Ca\(^{2+}\) concentration is maintained against high Ca\(^{2+}\) concentrations outside the cells and inside the various membrane systems. The regulation for maintaining the low cytoplasmic Ca\(^{2+}\) is thought to involve both the accumulation of Ca\(^{2+}\) in subcellular organelles, in particular mitochondria, through an active Ca\(^{2+}\) transport system (8, 17) and an active extrusion through the plasma membrane (4).

For the accumulation of Ca\(^{2+}\) by plant mitochondria, energy can be supplied by either substrate oxidation or exogenously added ATP (7, 8). Oligomycin specifically inhibits the ATP-driven Ca\(^{2+}\) uptake, but not the substrate-dependent uptake, suggesting that a high energy intermediate of oxidative phosphorylation is the direct energy source for mitochondrial Ca\(^{2+}\) uptake in plants (8) and in animals (9). Roux et al. (17) demonstrated that the active Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) fluxes by phytochrome; however, in their system the active accumulation of Ca\(^{2+}\) 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 Ca\(^{2+}\) fluxes by plant mitochondria seem to be altered by light, through the action of phytochrome.

Most Ca\(^{2+}\) 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 Ca\(^{2+}\) fluxes and Ca accumulation can be followed during light/dark transitions. Variations in Ca\(^{2+}\) concentrations in both cytosol and mitochondria during light/dark transitions could be important in the regulation of Ca\(^{2+}\)-requiring enzymes.

In this paper, we show that an ATP-dependent Ca\(^{2+}\) 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 \(\mu\)E/m\(^2\)-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 Ca\(^{2+}\) as described previously (12).

Isolation of Mitochondria by Density Gradient Centrifugation. All reagents and buffer solutions were prepared with double distillation H\(_2\)O to minimize Ca\(^{2+}\) 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 aggre- gated 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 SW50-1 rotor at 150,000g for 1 h. 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 KCl, 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 mM murexide, 2.5 mM MgCl2, 5 mM K2HPO4, 10 mM KCl, 0.2 mM CaCl2, and a known amount of the 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 A440-407nm 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 N 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 equiva-}

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 less total Ca content in their mitochondrial fraction (Table II). To demonstrate whether the dark treatment resulted in a reduction of Ca either in the mitochondrial soluble matrix 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 phosphohyl 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-
Table 1. Rate of Ca\(^{2+}\) Uptake and Ca\(^{2+}\) Accumulation into Mitochondrial Fraction Isolated from Corn Shoots Grown in the Light or Dark

The mitochondrial Ca\(^{2+}\) uptake was monitored by \(A_{340-507}\) change with dual wavelength spectroscopy with 50 \(\mu\)M 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 \(\mu\)g 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)(^a)</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 (\mu)M)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\) 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 = \(24.3 \times 0.85 + 31.5 \times 0.15\), and this was divided by mg of mitochondrial protein contained. Protein content of the mitochondrial fraction was 381 and 315 \(\mu\)g 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></td>
<td>Succinate: Cyt c reductase</td>
<td>NADH-GDH</td>
</tr>
<tr>
<td></td>
<td>% of sonicated fraction</td>
<td>nmol (-)fraction(^{-1})</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonicated</td>
<td>100</td>
<td>56.9</td>
</tr>
<tr>
<td>Matrix</td>
<td>80</td>
<td>24.3</td>
</tr>
<tr>
<td>Membrane</td>
<td>15</td>
<td>31.5</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonicated</td>
<td>100</td>
<td>36.6</td>
</tr>
<tr>
<td>Matrix</td>
<td>85</td>
<td>20.5</td>
</tr>
<tr>
<td>Membrane</td>
<td>15</td>
<td>16.5</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 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 \textit{in vitro}, our data do not agree with the observations by Roux \textit{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 Marmé (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\textsuperscript{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\textsuperscript{2+} uptake (results not shown). Therefore, the involvement of calmodulin in the stimulation of ATP-dependent Ca\textsuperscript{2+} uptake system by light seems to be ruled out.

Acknowledgment—We thank Prof. T. Kawasaki of Okayama University for making available the Hitachi two-wavelength double beam spectrophotometer, model 356 and Aminco-Chem Glow photometer, model 14-7441.

LITERATURE CITED

2. Dieter P, D Marme 1980 Calmodulin activation of plant microsomal Ca\textsuperscript{2+} uptake. Proc Natl Acad Sci USA 77: 7311–7314