Action of Calcium on Corn Mitochondria

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Our laboratory has been investigating membrane-linked functions of plant mitochondria which might help explain active ion transport. Corn mitochondria will actively accumulate calcium + phosphate in a process very like that of animal mitochondria (9); i.e., a high energy intermediate derived from substrate oxidation or ATP furnishes the energy. With the substrate-powered system, there appears to be a competition between uptake and ATP formation, for the addition of ADP + hexokinase trap diminished calcium uptake. However, unlike animal mitochondria, the corn mitochondria proved to require phosphate for calcium uptake. Furthermore, the accumulation of magnesium and phosphate could not be demonstrated unless some calcium was present.

These experiments were done with low concentrations of calcium (0.1–0.2 mM) in order to avoid uncoupling the mitochondria. We had observed that concentrations of calcium of 1 mM and higher reduced P/O ratios, and we assumed that calcium was acting as an uncoupling agent, a view commonly expressed on the basis of experiments with mammalian mitochondria. Calcium has been long known to stimulate acceptorless respiration of animal mitochondria (16, 17) and to induce adenosine triphosphatase activity (13), thus mimicking the action of the uncoupling agent, 2,4-dinitrophenol (DNP). Calcium also accelerates the swelling of animal mitochondria, which is thought to reflect a calcium-stimulated release of fatty acids, or U-factor (10).

More recent work suggests that the action of calcium is more complex than initially visualized. Chance (2) has shown that low concentrations of calcium do not really uncouple; rather, calcium simulates the effect of ADP as an energy acceptor. In the presence of phosphate, calcium activates a rapid energy-linked process which stimulates respiration and highly oxidizes the respiratory carriers. This process terminates with the binding of calcium in a form that no longer stimulates respiration, and the carriers return to the reduced state. High concentrations of calcium damage the mitochondria, causing pyridine nucleotide to be highly oxidized and respiration to be inhibited. Rossi and Lehninger (15) have examined the stoichiometry of calcium uptake and respiration in rat liver mitochondria. They conclude that the release of acceptorless respiration by small amounts of calcium is associated with a reaction at energy conserving sites of the electron transport chain. No phosphate is required for the reaction, but only a small amount of calcium can be bound or accumulated. The stoichiometry of binding and respiration is abolished by phosphate, and complete uncoupling of respiration results. However, further addition of ATP + magnesium will lead to calcium + phosphate uptake. When calcium and ADP + phosphate are added, both accumulation of calcium + phosphate and phosphorylation of ADP occur. The affinity of calcium for the energy conserving sites appears to be higher than that of ADP.

There is very little comparable information on the effect of calcium on plant mitochondria. Calcium has been reported to both accelerate (5) and inhibit (14) adenosine triphosphatase. The swelling of cauliflower mitochondria is unaffected or slightly retarded by 1 mM calcium (11). Hackett (6) found calcium and other divalent ions to accelerate the NADH-oxidase of sweet potato mitochondria.

It seemed to us that before we went further in studies of calcium + phosphate uptake we should study the effect of calcium on other energy-linked processes in corn mitochondria. The experiments are reported here, and comparisons are made with the action of 2,4-dinitrophenol (DNP). Contrary to our initial assumption, we find that calcium is not a true uncoupling agent for corn mitochondria. Calcium does not mimic the action of DNP as it does with animal mitochondria. However, calcium definitely reacts with corn mitochondria. At concentrations of 1 mM or less, calcium diverts phosphate from ATP formation to phosphate uptake and promotes the contraction of swollen mitochondria. Concentrations of calcium above 1 mM are very inhibitory to electron transport and phosphorylation.

Materials and Methods

Mitochondria were isolated from 3 and one-half-day etiolated maize shoots (WF9 × M14) essentially by methods previously described (7, 8). About 100 g of shoots were ground in an ice cold mortar with about 250 ml of 0.5 M sucrose + 5 mM EDTA + 0.05 M KH₂PO₄, with pH adjusted to 7.5 with Tris. In some experiments on oxidative phosphorylation the KH₂PO₄ was omitted, and the pH maintained with 0.1 M Tris chloride or maleate. No differences at-
tributable to buffer used were ever noted. The homogenate was strained through cheesecloth and centrifuged at 2000 × g for 10 minutes in a refrigerated centrifuge at 0° to remove nuclei and cell debris. Mitochondria were isolated from the supernatant at 10,000 × g for 10 minutes, and were washed in 0.5 m sucrose + 5 mM EDTA. Final suspension was in 0.5 m sucrose.

O₂ consumption associated with studies of phosphorylation was determined with the Warburg respirometer at 30°. Disappearance of inorganic phosphate was measured over the period of O₂ consumption on aliquots of the reaction medium cleared of mitochondria by 5% trichloroacetic acid or by centrifugation at 20,000 × g for 10 minutes. The vessel contained in 2.5 ml of medium 250 μmoles sucrose, 100 μmoles glucose, 50 μmoles KH₂PO₄, 40 μmoles each of pyruvate and malate, 2.5 μmoles MgSO₄, 6.2 μmoles ATP, 0.6 μmole NAD, 0.4 μmole thiamine pyrophosphate, 0.1 μmole CoA, 25 KM units hexokinase, and about 0.1 mg mitochondrial N (final pH 7.5).

The same medium was used in studying phosphate accumulation, with the amount of mitochondria increased to about 0.25 mg N. Incubation in centrifuge tubes at 30° for 30 minutes was followed by cooling in crushed ice and centrifugation of the mitochondria through 5 ml of 0.5 m sucrose layered beneath the mixture. Similar mixtures which were not incubated were used as initials. The supernatant solution was decanted, mixed, and analyzed for phosphate. Mitochondrial phosphate was determined by a modification of the method of Weil-Malherbe and Green (21). The mitochondrial pellet was directly extracted with vigorous shaking for 15 seconds with 3 ml of 1.13 × H₂SO₄, 1 ml of 5% ammonium molybdate and 6 ml of isobutanol. The tubes were centrifuged for 30 seconds at about 1000 × g in a bench centrifuge and 3 ml of the isobutanol layer analyzed for phosphate with the stannous chloride reagent as described by Weil-Malherbe and Green.

The adenosine triphosphatase activity was determined by Pi released from ATP during 30 minutes incubation at 28°. The basic medium consisted of 0.2 m KCl, 0.02 m Tris (pH 7.5), 5 mM ATP and 1 mM MgSO₄ with CaCl₂ additions as noted. The reaction was started by addition of the mitochondria, and stopped with cold 5% trichloroacetic acid. Initial aliquots were taken immediately after adding the mitochondria.

Swelling and contraction were followed by OD changes at 520 m, in a Coleman Model 11 spectrophotometer. A comprehensive study by Stoner (18) shows the change in OD is correlated with water content and configurational changes of the inner membrane (manuscript in preparation). Swelling was initiated by rapidly mixing 0.1 ml of mitochondria into 4.9 ml of 0.1 m KCl + 0.02 m Tris (pH 7.5) with the indicated concentrations of CaCl₂. Solutions were maintained in a 28° water bath between readings. Contraction was initiated with 3 mM ATP, 6 mM MgCl₂ and 2 mg/ml bovine serum albumin (BSA), or with 10 mM succinate (19). Corrections were made for the OD change accompanying dilution.

Polarographic determinations of O₂ consumption were made according to Wiskich and Bonner (22), using a Clark oxygen electrode. The mitochondria were isolated in the sucrose-EDTA-Phosphate-Tris medium as above, washed once in the sucrose-EDTA, and finally in 0.5 m sucrose to minimize residual buffer and EDTA. Final sucrose suspension contained 0.18 to 0.2 mg mitochondrial N in 0.1 ml. The reaction medium was the same as that used in the Warburg experiments with the omission of ATP, glucose, and hexokinase, and with the addition of 1 mg BSA/ml and 0.01 m tris (pH 7.5). Phosphate was a variable as noted with the data. Reaction was started by addition of 0.1 ml of mitochondrial suspension to 2.5 ml of medium. Further additions were made in small volumes, generally 0.01 ml, from stock solutions of ADP, DNP or CaCl₂. During the course of the experiments the mitochondria were held on ice in a thin layer on the bottom of a beaker in order to provide better aeration.

**Results**

**Adenosine Triphosphatase and Oxidative Phosphorylation.** Figure 1 presents the response obtained
in studies of adenosine triphosphatase and oxidative phosphorylation as affected by calcium. The adenosine triphosphatase determined under these conditions is largely associated with contraction or maintenance of contraction; it is accelerated by exogenous magnesium, but can apparently proceed to some degree with endogenous magnesium; it is oligomycin-sensitive (18). The point of importance here is that calcium did not accelerate the adenosine triphosphatase, and in millimolar concentrations began to depress it. Dinitrophenol, however, stimulates the adenosine triphosphatase of corn mitochondria under these conditions (19).

Similarly, there was no important effect of calcium on P/O ratios at concentrations below 1 mm. Indeed, at 1 mm calcium there was greater depression of respiration (32 %) than of coupling (13 %). Quite unlike the situation with dinitrophenol where P/O ratios decline before respiration is significantly affected. (In a comparable experiment, 0.1 mm DNP reduced phosphorylation by 38 %, O₂ consumption by 9 %.)

The pronounced decline in P/O at calcium concentrations above 1 mm will not be considered here. Since the reduced P/O was associated with considerable depression of substrate oxidation and/or electron transport, it is probable that the mitochondria were damaged as suggested by Chance (2) and others. We can offer no sound explanation for the reduced respiration. What appears to be the same inhibitory effect can be secured with a number of divalent ions, zinc and copper being particularly effective. The amount of calcium needed to produce pronounced damage varied from about 2 to 5 mm, and the extent of damage appeared to increase with time.

In experiments like those of figure 1, phosphorylation was estimated by disappearance of inorganic phosphate from the medium. It was found in preliminary investigations that if the mitochondria were removed by centrifugation, rather than by acid precipitation, higher P/O values were obtained from treatments containing calcium (table I). Apparentlly, in the presence of calcium some inorganic phosphate is accumulated by the mitochondria. The acid precipitation extracts this phosphate, resulting in a lower P/O ratio than with centrifugation.

Verification of this result was sought by analysis of the mitochondria for inorganic phosphate (table II). For these experiments, 1 mm calcium was used as the minimum concentration which would lower the P/O ratio significantly without depressing respiration any more than necessary. There is unquestionably an uptake of phosphate by the mitochondria in the presence of calcium, and a small loss in its absence. Note that these mitochondria were in complete medium with a hexokinase trap, just as in

Table II. Calcium-Induced Phosphate Uptake in Corn Mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Δ P_i solution (μmoles)</th>
<th>Δ P_i mitochondria (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-24.1 ± 1.24</td>
<td>-0.6 ± 0.05</td>
</tr>
<tr>
<td>+ CaCl₂ (1 mm)</td>
<td>-15.7 ± 2.65</td>
<td>+3.1 ± 0.29</td>
</tr>
</tbody>
</table>

Initial and final 2 ml aliquots of the reaction medium from paired Warburg vessels were cleared of mitochondria by precipitation with ice cold 5% trichloroacetic acid, or by centrifugation at 0° for 10 minutes at 20,000 × g. The disappearance of Pi was determined.
figure 1 and table I. Making due allowance for the depression of O_2 consumption at 1 mM calcium (25-30 %, fig 1), we have calculated that there is no real decline in P/O provided one equates the phosphate accumulated with that esterified to glucose via ATP. Calcium appears to have diverted some portion of the phosphate normally passing to ATP into phosphate uptake.

Swelling and Contraction. The effect of calcium and DNP on swelling and contraction is shown in figure 2 and table III. As previously reported (19),

Table III. Promotion of Contraction by Calcium

<table>
<thead>
<tr>
<th>Additive</th>
<th>% Swelling</th>
<th>% Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25.5</td>
<td>63.4</td>
</tr>
<tr>
<td>CaCl_2 0.1 mM</td>
<td>25.3</td>
<td>73.7</td>
</tr>
<tr>
<td>None</td>
<td>27.3</td>
<td>57.0</td>
</tr>
<tr>
<td>CaCl_2 1.0 mM</td>
<td>24.5</td>
<td>64.6</td>
</tr>
<tr>
<td>KHPO_4 1 mM</td>
<td>23.3</td>
<td>70.3</td>
</tr>
<tr>
<td>+ CaCl_2 0.1 mM</td>
<td>24.3</td>
<td>85.5</td>
</tr>
<tr>
<td>+ CaCl_2 0.3 mM</td>
<td>24.0</td>
<td>95.4</td>
</tr>
<tr>
<td>+ CaCl_2 1.0 mM</td>
<td>22.7</td>
<td>92.1</td>
</tr>
</tbody>
</table>

The action of uncoupling agents like DNP is to accelerate the rate of attainment of the swollen state, and to reduce the level of contraction attained. Millimolar calcium produced the opposite effect: the rate and extent of swelling were retarded and the final level of contraction was slightly higher. No significant effect on swelling could be found with 0.1 mM calcium, although there was a consistent small increase in contraction. In the presence of 1 mM Pi, calcium had a pronounced effect in stimulating contraction (table III, fig 3), both with ATP-powered contraction where Pi is not inhibitory, and with succinate-powered contraction where phosphate is very inhibitory (18, 19). Figure 3 shows a 2-phase contraction in the presence of phosphate and the absence of calcium. This is an extreme example; more often the phases are not so pronounced, but are usually noticeable. The phenomenon has not been investigated as yet. Neither has the decline in succinate-powered contraction with time in the presence of calcium. The decline is greater with higher concentrations of calcium.

No detailed inquiry has been made as yet into the action of calcium in augmenting the level of contraction. For the purpose of this investigation, the important point is that calcium does not act like an uncoupling agent in the swelling and contraction of corn mitochondria. Nor did it do so in the swelling experiments of Lyons and Pratt (11) with cauliflower mitochondria. It should be noted that the plant mitochondria swell spontaneously at a much more rapid rate than do rat liver mitochondria (11). Perhaps calcium potentiates changes in liver mitochondria which occur spontaneously in isolated plant mitochondria.

Oxygen Electrode Experiments. Corn mitochondria proved to possess reasonably good respiratory control. Except for the final washing with sucrose to minimize phosphate and EDTA, no special isolation procedures were needed. With freshly isolated mitochondria the state 3/state 4 values were as high as 4, and ADP/P ratios were about 3. Figure 4A illustrates this, and shows the highest respiratory stimulation found with calcium, about 80%. Under these conditions (phosphate and ATP present) the stimulation persisted undiminished for as long as 4 minutes, the longest we followed it, and the calcium did not interfere with subsequent state 3 to state 4 transition on addition of ADP.

As is also shown in figure 4A, DNP produced a greater stimulation of respiration than did calcium,
Fig. 4. Tracings of recorder chart made in experiments with the oxygen electrode. Mitochondria (0.18 mg N) added at M to basic mixture without acceptor. Other additions as indicated at arrows. Temperature 22°. Rates are given as μatoms O₂/minute. Basic mixture consisted of 2.5 ml of medium containing 250 μmoles sucrose, 40 μmoles each pyruvate and malate, 25 μmoles Tris-Cl (pH 7.5), 2.5 μmoles MgSO₄, 0.6 μmole NAD, 0.4 μmole thiamine pyrophosphate, 0.1 μmole CO₂, and 0.45 μmole EDTA.
approaching the state 3 rate with ADP. The uncoupling of respiration by DNP with and without phosphate but in the absence of ATP and calcium is shown in figure 4E and F. It was assumed that the 0.04 mm concentration of DNP would be about optimal as it is for potato mitochondria (22).

When 0.4 mm calcium was added to a system lacking phosphate and ATP, the respiratory rate would immediately rise, then slowly decline (fig 4B). This is somewhat similar to the result reported by Rossi and Lehninger (15), but the transition between stimulated and basal respiration is not nearly so distinct. Furthermore, lower concentrations of calcium (0.16 mm) in the range used by these authors did not produce any marked effect (fig 4C). Subsequent addition of phosphate did not produce an uncoupled respiration. The exposure to calcium prior to addition of phosphate must have damaged the mitochondria for they lost respiratory control and responded only weakly to ADP and DNP (fig 4B). When phosphate was present initially, the addition of calcium had no effect on respiration or on subsequent respiratory control (fig 4D). This might have been due to lowering of the effective calcium concentration by the phosphate. However, even with 1 mm phosphate uncoupling by calcium as described by Rossi and Lehninger was never observed.

**Discussion**

It is evident that calcium is not an uncoupling agent for corn mitochondria, at least not in the same sense that dinitrophenol is an uncoupling agent. Specifically, calcium differs from DNP in that it does not promote swelling, it does not promote adenosine triphosphatase, it does not produce a sustained release of acceptorless respiration. There is a small sustained increase in acceptorless respiration in the presence of phosphate and ATP (fig 4A), but this is a condition leading to ion uptake (9), for which energy must be furnished. With phosphorylating preparations, the slight depression of P/O ratios up to millimolar concentrations of calcium (fig 1) should not be considered uncoupling. Calcium appears to divert energy from phosphorylation of ADP into phosphate uptake. Respiration is not really uncoupled; it is coupled to a different process, which is essentially the same conclusion reached with animal mitochondria (2, 15). (Again, the lowering of P/O which accompanies suppression of respiration is here considered to be a separate phenomenon associated with some unspecified damage, and will not be further considered.)

Only in the respiratory stimulation and decline in the absence of phosphate and ATP (fig 4B) is there evidence of an uncoupling reaction comparable to that reported for liver mitochondria (15). Even this action of calcium must be different in some respect, for it does not produce the sharp stimulation and decline found with liver mitochondria (i.e., the type of response produced by ADP in fig 4A), and it does not lead to complete uncoupling in the presence of phosphate. It simply must be accepted that there are some differences between corn and liver mitochondria, and we do not yet have sufficient knowledge to analyze these. The most important difference is that calcium produces responses in liver mitochondria which are already activated in corn mitochondria. Rapid swelling is spontaneous in corn, and the adenosine triphosphatase is evidently not latent. The effects of calcium on corn mitochondria must then be in less dramatic activities, activities which may be masked in animal mitochondria by their pronounced respiratory, adenosine triphosphatase and swelling responses.

Taking a more positive view, what does calcium do to corn mitochondria? The only effects of significance lie in what appears to be a diversion of energy into contraction, particularly in the presence of exogenous phosphate (table III and fig 3), and into phosphate uptake (tables 1 and II). And how is this accomplished? Little can be said about the contraction at present. Millimolar calcium has no important effect on ATP-powered contraction except when calcium is added (table III). Succinate-powered contraction is strongly inhibited by 1 mm phosphate (18, 19), and the action of calcium (fig 3) can be viewed as a release of the inhibition. Experiments now in progress show the promotive effect of calcium on contraction to be associated with phosphate uptake. Phosphate is not taken up in the absence of calcium. There is thus some relationship between calcium phosphate transport and contraction which we have not fully investigated. The most that can be said at this time is that the mechanism of calcium-activated phosphate uptake produces conditions conducive to good contraction, but that the uptake of phosphate (or calcium) is not essential to contraction.

The action of calcium in diverting respiratory energy into phosphate uptake (tables I and II) is of basic interest. Previous work with corn mitochondria has shown phosphate uptake to depend upon calcium (9). Conversely, no calcium uptake was found without phosphate. It was deduced that the uptake was at the expense of a high-energy phosphorylated intermediate of oxidative phosphorylation. If this view is to be maintained, we must assume that calcium participates as a cofactor in a transport mechanism for phosphate, the phosphate being derived ultimately from the phosphorylated intermediate. (See the scheme in fig 7, ref. 9; the question mark represents the unknown calcium-activated phosphate transport process.) In other words, corn mitochondria may be actively transporting phosphate, not calcium; calcium would serve as a cofactor and accompanying cation. However, a comparable diversion of energy into phosphate uptake could be obtained by a binding reaction of calcium to a non-phosphorylated intermediate at coupling sites, fol-
followed by a discharge inwards with Pi as an appropriate accompanying anion. As an energy-expending act, that is, an "uncoupling" act, the uptake process would lower ATP formation. Phosphorylation of the high energy intermediate would not be an essential part of the mechanism. [There may be an ATP requirement as described by Rossi and Lehninger (15) since calcium uptake by corn mitochondria is promoted by, but not dependent upon, exogenous ATP (9). We explained this on a mass action basis, but it is not excluded that endogenous ATP may play some essential role.]

This latter view would be more nearly in line with the conclusions of Rossi and Lehninger discussed above. Chappell et al. (3) and Brierley et al. (1) report that manganese and magnesium, respectively, are bound by mitochondria in the absence of phosphate by an energy dependent act associated with release of hydrogen ion. Millard et al. (12), working with beet root mitochondria, have hypothesized that cation binding to active centers which release hydrogen ion is the fundamental mechanism of ion accumulation.

Of the two alternatives, then, most experiments and opinion would support the view that the calcium-induced diversion of phosphate into uptake observed here is a consequence of calcium binding at energy conserving sites. This is followed by active accumulation of calcium accompanied by Pi, consuming energy which would otherwise be used in ATP formation. However, we cannot fully confirm the basic phenomenon on which the view is predicated, that is, calcium acting as an uncoupling agent. If it were, it should at least act like DNP in those systems where no acceptor is involved. Since it does not, there is no sound experimental basis for assuming that calcium reacts directly at energy conserving sites in corn mitochondria. The reaction may occur, but it does not produce any pronounced manifestations of uncoupling, such as release of acceptorless respiration or latent adenosine triphosphatase.

We still think it is possible that the phosphate accumulated by corn mitochondria is derived from a phosphorylated intermediate by some transfer mechanism which utilizes calcium. The transfer mechanism could even be the intermediate system involved in coupling electron flow to phosphorylation: the only distinction would be that in corn the intermediate would not be discharged and recycled for further electron flow, except after reaction with phosphate to form the phosphorylated intermediate. Calcium could then be visualized as mediating a shunt mechanism, competitively reducing the high energy phosphate available for ATP formation. It is worth emphasizing that in previous work (9) repeated attempts were made to obtain calcium uptake without phosphate and all failed. These experiments were with low concentrations of calcium, and if the phenomenon were as described for liver mitochondria, some active calcium binding should have been noted.

Summary

The action of calcium on corn mitochondria has been investigated. No evidence for uncoupling of phosphorylation could be found unless concentrations of calcium were used which strongly suppressed respiration. Lower concentrations of calcium did lower P/O ratios, but this is due to diversion of phosphate from ATP formation into uptake. Calcium did not stimulate adenosine triphosphatase or swelling rates. Contraction with ATP or succinate was enhanced by calcium, particularly in the presence of phosphate. Only small stimulations of acceptorless respiration were found; these were not sustained except in the presence of phosphate and ATP. In no respect did calcium act like 2,4-dinitrophenol. The action of calcium in diverting phosphate into uptake is discussed.

Acknowledgment

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Literature Cited


