

# Effects of Low Temperature and Respiratory Inhibitors on Calcium Flux in Plant Mitochondria

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## ABSTRACT

The effects of low temperature on uptake and release of <sup>45</sup>Ca<sup>2+</sup> were studied with sound, well-coupled mitochondria extracted at room temperature from avocado (*Persea americana* Mill, cv Fuerte) fruits. Low Ca<sup>2+</sup> concentrations (10 micromolar) were employed to simulate physiological conditions. At 25°C, the rate of Ca<sup>2+</sup> uptake decreased with time, whereas at 5°C the initial rate, though lower, remained linear. As a consequence total uptake at 5°C was substantially greater than at 25°C for periods greater than 5 min. Preincubation of mitochondria at 5°C enhanced subsequent Ca<sup>2+</sup> uptake at 25°C. Ca<sup>2+</sup> uptake was inhibited by carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) and by ruthenium red, but neither KCN nor salicylhydroxamic acid separately or together had any major inhibitory effect. Preloaded mitochondria held for 60 min in a Ca-free medium lost little Ca<sup>2+</sup> at 25°C and none at 5°C, except in the presence of ruthenium red or CCCP.

The response of mammalian cells to external stimuli is frequently mediated by transient changes in the cytoplasmic Ca<sup>2+</sup> concentration, often via interaction with Ca-binding proteins such as calmodulin (5). Normal cell functions require that cytoplasmic Ca<sup>2+</sup> be rigorously maintained at concentrations below 1 μM, and nanomolar changes in Ca<sup>2+</sup> concentration can initiate major metabolic events.

The recent identification of calmodulin in plant cells indicates potential for similar control of metabolism by modulation of Ca<sup>2+</sup> concentrations (1, 7). Such a mechanism has been suggested for some phytochrome responses and for the regulation of cytoplasmic streaming (24). In a study of cells sensitive to chilling temperatures, Woods *et al.* (26) have found evidence suggesting that changes in cyclosis induced by low temperature may result from elevated cytoplasmic Ca<sup>2+</sup> concentrations.

Because of the central role of mitochondria in the regulation of cytoplasmic Ca<sup>2+</sup> in mammalian cells (3, 13), it is reasonable to ask whether plant mitochondria have a similar function. It is well known that the uptake of Ca<sup>2+</sup> by plant mitochondria is energy-dependent (6, 8, 9). It has also been shown that respiration of mitochondria from chilling-sensitive plant tissue is perturbed below the threshold temperature for chilling (15). We were therefore interested to determine whether the uptake and release of Ca<sup>2+</sup> by mitochondria might be impaired by their exposure to low temperature.

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## MATERIALS AND METHODS

**Plant Material.** Avocado fruits (*Persea americana* Mill, var Fuerte) were obtained from the University of California South Coast Field Station. The fruits were ripened by placing them at 20°C in a flowing air stream containing 10 μl/l ethylene.

**Preparation of Mitochondria.** Mitochondria were isolated from avocado fruits as described by Ozelkok and Romani (17), except that all operations including centrifugation were performed at room temperature (22 ± 2°C). The isolation medium contained increased concentrations of PVP (0.6%), BSA (0.3%), and β-mercaptoethanol (15 mM). A respiratory substrate (2 mM α-ketoglutarate) was also added. When not used immediately, the isolated mitochondria were held at room temperature in incubation medium. Their RCR<sup>2</sup> was checked before use to confirm that functional integrity had been maintained.

The incubation and assay medium used with isolated mitochondria contained 0.25 M sucrose, 0.1% BSA, 50 mM phosphate buffer (pH 7.2), 1 mM MgCl<sub>2</sub>, 10 μM CoA, 100 μM thiaminepyrophosphate, 100 μM NAD, 0.01% chloramphenicol, 0.05 μM ADP, substrate (10 mM α-ketoglutarate) was added as shown.

**Mitochondrial O<sub>2</sub> Uptake and Protein Determination.** O<sub>2</sub> uptake was measured at 25°C with a Clark-type polarographic electrode. Protein contents were measured by the Lowry procedure (16).

**Ca<sup>2+</sup> Uptake and Release.** Mitochondria (approximately 2 mg protein ml<sup>-1</sup>) were gently agitated at 25°C in incubation medium containing 10 μM CaCl<sub>2</sub> including 3.7 × 10<sup>3</sup> Bq·ml<sup>-1</sup> of <sup>45</sup>Ca<sup>2+</sup>. At intervals, 100-μl samples were removed, filtered, and quickly washed twice on 0.45-μm Millipore filters with 1 ml of wash medium. Experiments showed that little additional Ca<sup>2+</sup> was released by further washes. The filters were placed in vials with Aquasol scintillant and radioactivities measured.

To study Ca<sup>2+</sup> release, mitochondria were preloaded with <sup>45</sup>Ca<sup>2+</sup> for 60 min and then centrifuged at 8000g for 5 min, and the pellet was washed twice by resuspending in 4 ml of incubation solution and recentrifuging. About 20% of the mitochondrial protein was lost during this centrifugation and washing procedure. The final pellet was resuspended in incubation medium and the release of <sup>45</sup>Ca<sup>2+</sup> from the mitochondria was followed by filtration and counting as described above.

For examination of uptake and release at low temperatures, the incubation vessels were agitated in an ice bath, providing an average incubation temperature of 5°C.

## RESULTS

**Mitochondrial Activity.** Isolation from avocado fruit at room temperature provided mitochondria with respiratory activity and

<sup>2</sup> Abbreviations: RCR, respiratory control ratio; RR, ruthenium red; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; SHAM, salicylhydroxamic acid; Ψ, membrane potential.

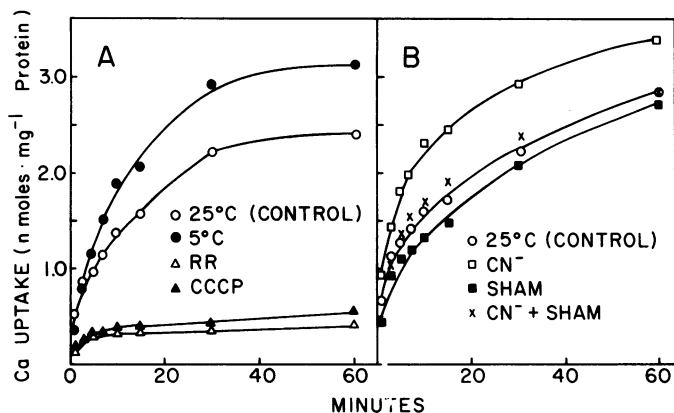


FIG. 1. Calcium uptake by avocado mitochondria. A, Uptake at 25°C; 5°C; 25°C + 10  $\mu$ M CCCP or 25°C + 20  $\mu$ M ruthenium red. B, Uptake at 25°C; + 1 mM KCN; + 2 mM SHAM or + KCN and SHAM.

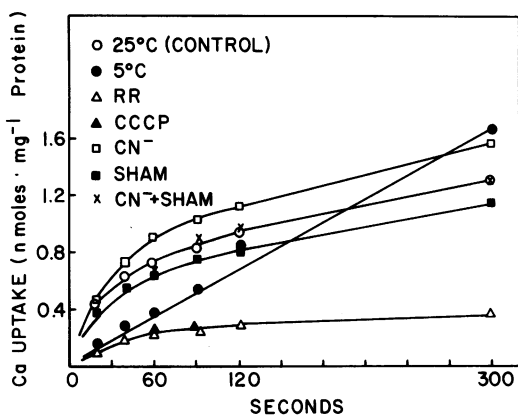


FIG. 2. Calcium uptake by avocado mitochondria over 5 min. Uptake at 25°C; + 20  $\mu$ M ruthenium red; + 10  $\mu$ M CCCP; + 1 mM KCN; + 2 mM SHAM; + KCN + SHAM; and uptake at 5°C.

Table I. Effect of Inhibitors on  $Ca^{2+}$  Uptake by Avocado Mitochondria at 25° and 5°C

Values are the mean rate over the first 5 min of uptake. Inhibitor concentrations are given in the text.

	Rate of $Ca^{2+}$ Uptake					
	Control	RR	CCCP	KCN	SHAM	KCN + SHAM
	<i>nmol·mg<sup>-1</sup> protein·5 min<sup>-1</sup></i>					
25°C	1.29	0.36 (28) <sup>a</sup>	0.37 (28)	1.59 (123)	1.15 (89)	1.30 (101)
5°C	1.60	0.15 (9)	0.21 (13)	1.64 (103)	1.12 (70)	0.83 (52)

<sup>a</sup> Numbers in parentheses, per cent of control.

respiratory control similar to that of mitochondria isolated at 4 to 5°C. Avocado mitochondria isolated in the cold have been shown to maintain respiratory control over several days (21) at room temperature if supplied with substrate and ADP. Mitochondria prepared at room temperature also maintained control for several hours, well beyond the experimental period. For example, in one experiment, the RCR was initially 3.0, and then 3.2, 2.7, and 2.4 at the 4th, 6th, and 10th h, respectively. Incubation for up to 4 h at 5°C had no effect on subsequent respiratory activity at 25°C. The low concentration of  $Ca^{2+}$  used for uptake and release (10  $\mu$ M) also had no effect on respiration, although millimolar concentrations sometimes inhibited respiration slightly.

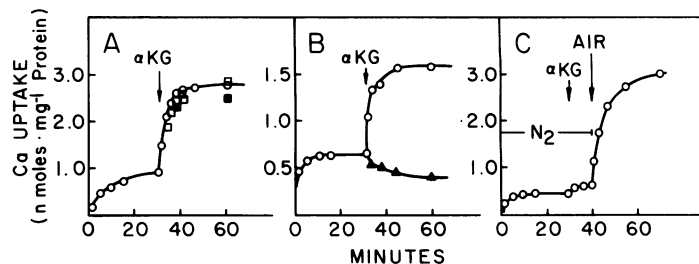


FIG. 3.  $Ca^{2+}$  uptake by avocado mitochondria after substrate depletion. Mitochondria were incubated at 25°C incubation medium minus substrate. At time zero, 10  $\mu$ M  $^{45}CaCl_2$  was added and uptake followed for 30 min. A,  $Ca^{2+}$  was added at  $t_0$  after 4 h incubation without substrate. Arrow indicates addition of 10 mM  $\alpha$ -ketoglutarate. Control (O); 1 mM KCN (□); 1 mM KCN, and 2 mM Sham (■). B,  $Ca^{2+}$  was added at  $t_0$  after 90 min incubation without substrate. Arrow indicates addition of 10 mM  $\alpha$ -ketoglutarate. Control (O); 10  $\mu$ M CCCP (▲). C,  $Ca^{2+}$  was added at  $t_0$  after 4 h incubation without substrate.  $Ca^{2+}$  uptake for 30 min, and then 10 min after the addition (first arrow) of 10 mM  $\alpha$ -ketoglutarate, was followed under  $N_2$ .  $N_2$  was replaced by air (second arrow) at 40 min.

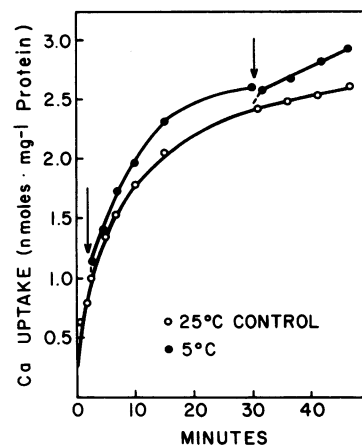


FIG. 4.  $Ca^{2+}$  uptake by avocado mitochondria at 25°C (O) and after transfer to 5°C at 2 min (first arrow) or 30 min (second arrow) (●).

Table II. Release of Preloaded  $^{45}Ca^{2+}$  from Avocado Mitochondria

Treatment	$Ca^{2+}$ Release			
	15 min	60 min	15 min	60 min
	<i>nmol·mg<sup>-1</sup> protein</i>		% of initial <sup>a</sup>	
25°C control	0.08	0.06	4.60	3.8
5°C	0.00	0.00	0.0	0.0
CCCP	1.21	1.41	74.8	87.6
Plus RR				
25°C control	0.46	0.55	28.9	33.9
5°C	0.24	0.33	14.9	20.4
CCCP	0.69	0.99	42.5	61.2

<sup>a</sup>  $Ca^{2+}$  content of preloaded mitochondria = 1.609 nmol  $mg^{-1}$  protein.

**$Ca^{2+}$  Uptake by Avocado Mitochondria.** Uptake of  $^{45}Ca^{2+}$  from a solution containing 10  $\mu$ M  $CaCl_2$  was rapid during the first few minutes, and approached a plateau usually about 60 min after the addition of  $Ca^{2+}$  (Figs. 1, 3, and 4). In short-term experiments at 25°C, up to 25% of the total  $Ca^{2+}$  accumulated in 1 h had already been taken up within 20 s (Fig. 2). Total uptake at 60 min was inhibited at least 75% by the addition of 20  $\mu$ M RR, the inhibition being evident at 20 s (Figs. 1A and 2).

Our use of a low (10  $\mu$ M)  $Ca^{2+}$  concentration is a deliberate

attempt to simulate physiological conditions. The low  $\text{Ca}^{2+}$  levels may preclude kinetic interpretation during long term incubations but, as seen below, did not prevent a positive response to KCN or 5°C treatment. Moreover, 10  $\mu\text{M}$  avoided the effects on coupling and respiratory activity generally observed with millimolar concentrations of  $\text{Ca}^{2+}$ .

**Effect of Respiratory Inhibitors.**  $\text{Ca}^{2+}$  uptake was substantially reduced (about 80%) by the uncoupler CCCP, the residual uptake being similar to that observed in the presence of RR (Figs. 1A and 2; Table I). In contrast, 1 mM KCN consistently increased  $\text{Ca}^{2+}$  uptake, this being evident before 60 s (Fig. 2) with a total enhancement (Fig. 1B) very similar to that achieved with low temperature (see below). SHAM (2 mM) slightly reduced uptake and the combined effects of KCN and SHAM generally negated each other (Table I) at 25°C.

Respiration was only partially inhibited by 1 mM KCN,  $\text{O}_2$  consumption being inhibited by an average of 56% in five different experiments. The combination of KCN and SHAM still left a substantial (about 20%) residual respiration. For example, in one experiment the initial state 3  $\text{O}_2$  consumption of 65 nmol  $\text{O}_2 \cdot \text{mg}^{-1} \text{protein} \cdot \text{min}^{-1}$ , was reduced to 29 with KCN and further to 15 with SHAM.

When mitochondria were placed in the incubation medium in the absence of substrate for up to 4 h to reduce  $\Psi$  (20) prior to the addition of  $\text{Ca}^{2+}$ , subsequent  $\text{Ca}^{2+}$  uptake was much reduced (Fig. 3) to the levels similar to those in the presence of CCCP (Figs. 1 and 2). Uptake was immediately stimulated upon addition of 10 mM  $\alpha$ -ketoglutarate, the rate increasing almost 100-fold (Fig. 3, A and B). A similar stimulation was achieved with 2 mM NADH as substrate (data not shown). The stimulation was unaffected by KCN or by KCN plus SHAM (Fig. 3A) and prevented by CCCP (Fig. 3B). Under both a  $\text{N}_2$  atmosphere and substrate depletion, the addition of  $\alpha$ -ketoglutarate caused only a minor stimulation in  $\text{Ca}^{2+}$  uptake. However, upon the introduction of air, an immediate stimulation occurred (Fig. 3C).

**Effect of Low Temperature.**  $\text{Ca}^{2+}$  uptake at 5°C followed quite a different pattern from that at 25°C. The initial uptake by the chilled mitochondria was depressed but remained linear for the first 5 min (Fig. 2). As a consequence, the total uptake after 60 min was substantially greater at 5°C than at 25°C (Fig. 1A). In three separate experiments, the average amount of  $\text{Ca}^{2+}$  accumulated per mg protein in 60 min was  $2.58 \pm 0.11$  at 25°C and  $3.33 \pm 0.19$  nmol at 5°C. Prior incubation of mitochondria at 5°C for 90 min followed by warming at 25°C for 30 min resulted in a  $\text{Ca}^{2+}$  uptake ( $3.69 \text{ nmol} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$ ) that was appreciably greater than in the control held at 25°C ( $2.80 \text{ nmol} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$ ), although the initial difference in linearity was not found. When mitochondria were transferred to 5°C 2 or 30 min after the start of  $\text{Ca}^{2+}$  uptake at 25°C, uptake was slightly enhanced (Fig. 4).

There was also some indication that chilling fruit prior to mitochondrial isolation had an effect on  $\text{Ca}^{2+}$  uptake. When preclimacteric fruit were chilled for 3 d at 1°C prior to the isolation of mitochondria at room temperature, the  $\text{Ca}^{2+}$  uptake rate was  $2.23 \text{ nmol} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$  as compared to  $0.87 \text{ nmol} \cdot \text{mg}^{-1} \text{protein} \cdot \text{h}^{-1}$  for fruit at an equivalent stage of ripening which had been stored at 15°C.  $\text{Ca}^{2+}$  uptake at 5°C was inhibited by RR and CCCP as at 25°C (Table I).

**$\text{Ca}^{2+}$  Release.** When mitochondria preloaded with  $^{45}\text{Ca}^{2+}$  were transferred to a minus- $\text{Ca}^{2+}$  medium at 25°C, a small proportion of  $\text{Ca}^{2+}$  was released (Table II). Release was more rapid and substantial in the presence of 20  $\mu\text{M}$  RR and greatly accelerated by CCCP. KCN and SHAM had little effect on release, (data not shown). Low temperature substantially suppressed  $\text{Ca}^{2+}$  release. There was no release at 5°C in the absence of RR and much less than at 25°C in the presence of RR.

In other experiments (data not shown), almost all (95%) of the

accumulated  $\text{Ca}^{2+}$  was released by incubating mitochondria with the  $\text{Ca}^{2+}$  ionophore A23187 (20  $\mu\text{M}$ ), and as much as 45% was released when harvested mitochondria were simply washed with 1 ml of the ionophore solution while on the Millipore filter. An initial pretreatment of the mitochondria with 5 mM EGTA at the end of the loading period removed about 40% of the accumulated  $\text{Ca}^{2+}$ , but did not substantially change the subsequent rate of release in a minus- $\text{Ca}^{2+}$  medium in the presence of RR.

## DISCUSSION

**Isolation of Mitochondria at Room Temperature.** Fully active mitochondria can be prepared from avocado fruit, ripe or unripe, at room temperature. Isolation under these conditions does not appear to impair subsequent mitochondrial activity or survival. This means that the effects of low temperature can be studied without the complications of low temperature pretreatment implicit in conventional isolation techniques. It may be that avocado fruit are particularly suited to room temperature isolation; their mitochondria have a remarkable homeostatic potential, being able to maintain respiratory control for several days *in vitro* (19, 21).

**$\text{Ca}^{2+}$  Uptake.** That  $\text{Ca}^{2+}$  uptake by plant mitochondria is energy-linked and phosphate dependent has been known for some time (10, 25). Though initially believed to be through a phosphorylated high energy intermediate (11), the phosphate-dependence is still not adequately accounted for (6). The only other study of avocado mitochondria and  $\text{Ca}^{2+}$  was by Chen and Lehninger (4) as part of a wider plant survey. They concluded that, while some tissues such as sweet potato yielded mitochondria with both high and low affinity binding sites and a significant capacity to accumulate  $\text{Ca}^{2+}$  coupled to electron transport, avocado mitochondria, along with those from a number of other species, had only low affinity sites and poor coupling to electron transport. They assumed that this resulted from a lack of specific carriers. Our results demonstrate that avocado fruit mitochondria accumulate  $\text{Ca}^{2+}$  at physiological concentrations as an energy-dependent process.

Inhibition of  $\text{Ca}^{2+}$  uptake by uncoupling protonophores such as CCCP has been found in many other studies with plant mitochondria. The inhibition of  $\text{Ca}^{2+}$  uptake by RR has also been found in mitochondria from corn (7, 18), mung bean, and potato (23). Both KCN and SHAM inhibitors, together or alone, inhibited  $\text{Ca}^{2+}$  uptake in mitochondria from corn, mung bean, and potato (7, 18, 23).

In contrast, our results clearly show that KCN actually stimulates uptake, and SHAM has only a slight inhibitory action. As suggested by a reviewer (anon.), the differential effects of KCN and SHAM when each is used alone could be accounted for by their modulation of phosphorylation, which is permitted by the presence of ADP and competes with ion transport for proton motive force. However, the lack of transport inhibition in the combined presence of KCN and SHAM following the addition of  $\alpha$ -ketoglutarate to substrate depleted mitochondria (Fig. 3A) suggests that  $\text{Ca}^{2+}$  uptake may be driven via residual respiration that is KCN + SHAM resistant and known to persist in avocado mitochondria (19). Alternatively, it is possible that KCN and SHAM inhibition is incomplete, allowing enough electron transport along normal channels to provide a driving force for  $\text{Ca}^{2+}$  uptake.

**$\text{Ca}^{2+}$  Release.** A question addressed by this work was whether mitochondria may be a source of increased cytosolic  $\text{Ca}^{2+}$  concentrations in response to low temperature. It is clear that low temperature *per se* will impede rather than stimulate  $\text{Ca}^{2+}$  release by avocado mitochondria *in vitro*. This observation does not preclude a low temperature-induced  $\text{Ca}^{2+}$  release *in vivo* where other and more complex controls may be operative. Mammalian mitochondria appear to release  $\text{Ca}^{2+}$  in an energy-linked process

which is independent of uptake and which is stimulated by several factors including an increase in reduced pyridine nucleotides (2, 14). Johnson and Wilson (12) have pointed out that the capacity of plant mitochondria to release previously accumulated  $\text{Ca}^{2+}$  indicates that they do not constitute permanent storage pools. Even the sequestering of calcium phosphate salts is not necessarily permanent (25). The  $\text{Ca}^{2+}$  uptake observed with avocado mitochondria is also reversible in that the accumulated  $\text{Ca}^{2+}$  can be released completely and rapidly by a  $\text{Ca}^{2+}$  ionophore or an uncoupler. Further study of  $\text{Ca}^{2+}$  release in plant mitochondria seems warranted.

**Low Temperature and  $\text{Ca}^{2+}$  Regulation.** The response to low temperature was unexpected, but consistent. Inhibitor effects (Table II) suggest that  $\text{Ca}^{2+}$  uptake at low temperature is energized in much the same way as that at  $25^\circ\text{C}$ . Total  $\text{Ca}^{2+}$  uptake was always greater at a low temperature and after low temperature pretreatment. Moreover, since efflux at the low temperature is less than that at  $25^\circ\text{C}$ , the overall effect is a decidedly greater net uptake of  $\text{Ca}^{2+}$  by mitochondria at the lower temperature. It would appear that if, in responding to low temperature, plant mitochondria are to influence the free  $\text{Ca}^{2+}$  levels in the cytosol, it would be via an increased conservation or sequestration of  $\text{Ca}^{2+}$ . A role for mitochondria in low temperature injury may lie in their energy-dependent removal of  $\text{Ca}^{2+}$  as part of a homeostatic response to low temperature stress.

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