Plant Physiol. (1985) 77, 877–880
0032-0889/85/77/0877/04/$01.00/0

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

Received for publication June 25, 1984 and in revised form December 12, 1984

IAN B. FERGUSON[1], MICHAEL S. REID, AND ROGER J. ROMANI*
Department of Environmental Horticulture and Department of Pomology, University of California, Davis, California 95616

ABSTRACT

The effects of low temperature on uptake and release of $^{45}Ca^{2+}$ were studied with sound, well-coupled mitochondria extracted at room temperature from avocado (*Persea americana* Mill, cv Fuerte) fruits. Low Ca^{2+} concentrations (10 micromolar) were employed to simulate physiological conditions. At 25°C, the rate of Ca^{2+} 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^{2+} uptake at 25°C. Ca^{2+} 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^{2+} 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^{2+} concentration, often via interaction with Ca-binding proteins such as calmodulin (5). Normal cell functions require that cytoplasmic Ca^{2+} be rigorously maintained at concentrations below 1 μM, and nanomolar changes in Ca^{2+} 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^{2+} 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^{2+} concentrations.

Because of the central role of mitochondria in the regulation of cytoplasmic Ca^{2+} 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^{2+} 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^{2+} by mitochondria might be impaired by their exposure to low temperature.

[1] Recipient of a Postharvest Biology Fellowship from the University of California. Present address: Division of Horticulture and Processing, Department of Scientific and Industrial Research, Private Bag, Auckland, New Zealand.

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[2] 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 $MgCl_2$, 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_2 Uptake and Protein Determination. O_2 uptake was measured at 25°C with a Clark-type polarographic electrode. Protein contents were measured by the Lowry procedure (16).

Ca^{2+} Uptake and Release. Mitochondria (approximately 2 mg protein ml^{-1}) were gently agitated at 25°C in incubation medium containing 10 μM $CaCl_2$ including 3.7×10^3 $Bq \cdot ml^{-1}$ of $^{45}Ca^{2+}$. 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^{2+} was released by further washes. The filters were placed in vials with Aquasol scintillant and radioactivities measured.

To study Ca^{2+} release, mitochondria were preloaded with $^{45}Ca^{2+}$ for 60 min and then centrifuged at 8000*g* 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 $^{45}Ca^{2+}$ 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

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

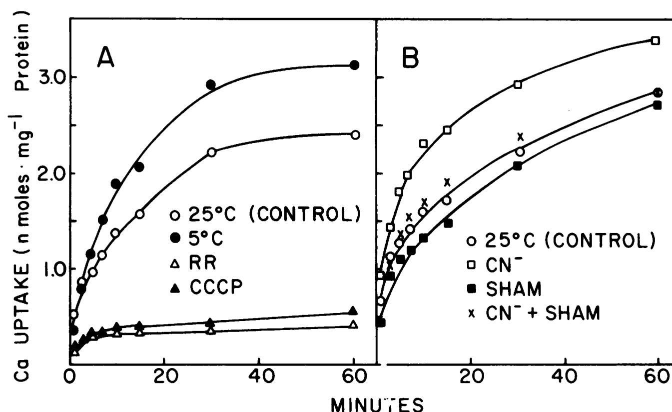

FIG. 1. Calcium uptake by avocado mitochondria. A, Uptake at 25°C; 5°C; 25°C + 10 μM CCCP or 25°C + 20 μM ruthenium red. B, Uptake at 25°C; + 1 mM KCN; + 2 mM SHAM or + KCN and SHAM.

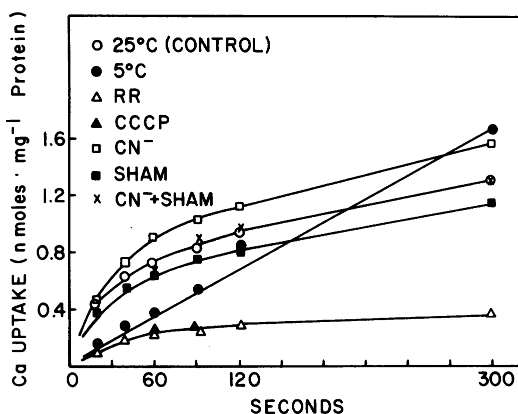

FIG. 2. Calcium uptake by avocado mitochondria over 5 min. Uptake at 25°C; + 20 μM ruthenium red; + 10 μM CCCP; + 1 mM KCN; + 2 mM SHAM; + KCN + SHAM; and uptake at 5°C.

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
	nmol·mg⁻¹ protein·5 min⁻¹					
25°C	1.29	0.36	0.37	1.59	1.15	1.30
		(28)[a]	(28)	(123)	(89)	(101)
5°C	1.60	0.15	0.21	1.64	1.12	0.83
		(9)	(13)	(103)	(70)	(52)

[a] 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 μM) also had no effect on respiration, although millimolar concentrations sometimes inhibited respiration slightly.

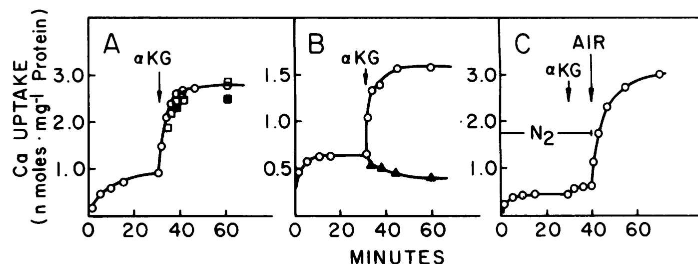

FIG. 3. Ca^{2+} uptake by avocado mitochondria after substrate depletion. Mitochondria were incubated at 25°C incubation medium minus substrate. At time zero, 10 μM $^{45}CaCl_2$ was added and uptake followed for 30 min. A, Ca^{2+} was added at t_0 after 4 h incubation without substrate. Arrow indicates addition of 10 mM α-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 α-ketoglutarate. Control (O); 10 μM 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 α-ketoglutarate, was followed under N_2. N_2 was replaced by air (second arrow) at 40 min.

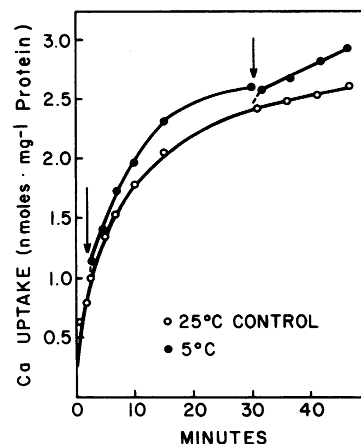

FIG. 4. Ca^{2+} uptake by avocado mitochondria at 25°C (O) and after transfer to 5°C at 2 min (first arrow) or 30 min (second arrow) (●).

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

Treatment	Ca^{2+} Release			
	15 min	60 min	15 min	60 min
	nmol·mg⁻¹ protein		*% of initial*[a]	
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

[a] 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 μM $CaCl_2$ 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 μM RR, the inhibition being evident at 20 s (Figs. 1A and 2).

Our use of a low (10 μM) Ca^{2+} concentration is a deliberate

attempt to simulate physiological conditions. The low 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 μM avoided the effects on coupling and respiratory activity generally observed with millimolar concentrations of Ca^{2+}.

Effect of Respiratory Inhibitors. 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 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, 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 O_2 consumption of 65 nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$, 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 Ψ (20) prior to the addition of Ca^{2+}, subsequent 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 α-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 N_2 atmosphere and substrate depletion, the addition of α-ketoglutarate caused only a minor stimulation in Ca^{2+} uptake. However, upon the introduction of air, an immediate stimulation occurred (Fig. 3C).

Effect of Low Temperature. 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 Ca^{2+} accumulated per mg protein in 60 min was 2.58 ± 0.11 at 25°C and 3.33 ± 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 Ca^{2+} uptake (3.69 $nmol \cdot mg^{-1}$ protein $\cdot h^{-1}$) that was appreciably greater than in the control held at 25°C (2.80 $nmol \cdot mg^{-1}$ protein $\cdot h^{-1}$), although the initial difference in linearity was not found. When mitochondria were transferred to 5°C 2 or 30 min after the start of 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 Ca^{2+} uptake. When preclimacteric fruit were chilled for 3 d at 1°C prior to the isolation of mitochondria at room temperature, the Ca^{2+} uptake rate was 2.23 $nmol \cdot mg^{-1}$ protein $\cdot h^{-1}$ as compared to 0.87 $nmol \cdot mg^{-1}$ protein $\cdot h^{-1}$ for fruit at an equivalent stage of ripening which had been stored at 15°C. Ca^{2+} uptake at 5°C was inhibited by RR and CCCP as at 25°C (Table I).

Ca^{2+} Release. When mitochondria preloaded with $^{45}Ca^{2+}$ were transferred to a minus-Ca^{2+} medium at 25°C, a small proportion of Ca^{2+} was released (Table II). Release was more rapid and substantial in the presence of 20 μM RR and greatly accelerated by CCCP. KCN and SHAM had little effect on release, (data not shown). Low temperature substantially suppressed 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 Ca^{2+} was released by incubating mitochondria with the Ca^{2+} ionophore A23187 (20 μM), 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 Ca^{2+}, but did not substantially change the subsequent rate of release in a minus-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).

Ca^{2+} Uptake. That 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 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 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 Ca^{2+} at physiological concentrations as an energy-dependent process.

Inhibition of Ca^{2+} uptake by uncoupling protonophores such as CCCP has been found in many other studies with plant mitochondria. The inhibition of 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 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 α-ketoglutarate to substrate depleted mitochondria (Fig. 3A) suggests that 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 Ca^{2+} uptake.

Ca^{2+} Release. A question addressed by this work was whether mitochondria may be a source of increased cytosolic Ca^{2+} concentrations in response to low temperature. It is clear that low temperature *per se* will impede rather than stimulate Ca^{2+} release by avocado mitochondria *in vitro*. This observation does not preclude a low temperature-induced Ca^{2+} release *in vivo* where other and more complex controls may be operative. Mammalian mitochondria appear to release 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 Ca^{2+} indicates that they do not constitute permanent storage pools. Even the sequestering of calcium phosphate salts is not necessarily permanent (25). The Ca^{2+} uptake observed with avocado mitochondria is also reversible in that the accumulated Ca^{2+} can be released completely and rapidly by a Ca^{2+} ionophore or an uncoupler. Further study of Ca^{2+} release in plant mitochondria seems warranted.

Low Temperature and Ca^{2+} Regulation. The response to low temperature was unexpected, but consistent. Inhibitor effects (Table II) suggest that Ca^{2+} uptake at low temperature is energized in much the same way as that as 25°C. Total 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°C, the overall effect is a decidedly greater net uptake of 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 Ca^{2+} levels in the cytosol, it would be via an increased conservation or sequestration of Ca^{2+}. A role for mitochondria in low temperature injury may lie in their energy-dependent removal of Ca^{2+} as part of a homeostatic response to low temperature stress.

LITERATURE CITED

1. ANDERSON JM, H CHARBONNEAU, HP JONES, RO MCCANN, MJ CORMIER 1980 Characterization of the plant nicotinamide dinucleotide kinase activator protein as calmodulin. Biochemistry 19: 3113–3120
2. BAUMHUTER S, C RICHTER 1982 The hydroperoxide-induced release of mitochondrial calcium occurs via a distinct pathway and leaves mitochondria intact. FEBS Lett 148: 271–275
3. CARAFOLI E, M CROMPTON 1978 The regulation of intracellular calcium by mitochondria. Ann NY Acad Sci 307: 269–284
4. CHEN C-H, AL LEHNINGER 1973 Ca^{2+} transport activity in mitochondria from some plant tissues. Arch Biochem Biophys 157: 183–196
5. CHEUNG WY 1980 Calmodulin plays a pivotal role in cellular regulation. Science 207: 19–27
6. DAY DA, BL BERTAGNOLLI, JB HANSON 1978 The effect of calcium on the respiration responses of corn mitochondria. Biochim Biophys Acta 502: 289–297
7. DIETER P, D MARME 1980a Calmodulin activation of plant microsomal Ca^{2+} uptake. Proc Natl Acad Sci USA 77: 7311–7314
8. DIETER P, D MARME 1980b Ca^{2+} transport in mitochondrial and microsomal fractions from higher plants. Planta 150: 1–8
9. EARNSHAW MJ, DM MADDEN, JB HANSON 1973 Calcium accumulation by corn mitochondria. J Exp Bot 24: 828–840
10. HANSON JB, TK HODGES 1967 Energy-linked reactions of plant mitochondria. Curr Top Bioenerg 2: 65–98
11. HANSON JB, BL BERTAGNOLLI, WD SHEPHERD 1972 Phosphate induced stimulation of acceptorless respiration in corn mitochondria. Plant Physiol 50: 347–354
12. JOHNSON HM, RH WILSON 1973 The accumulation and the release of divalent cations across mitochondrial membranes. Am J Bot 60: 858–862
13. LEHNINGER AL, E CARAFOLI, CS ROSSI 1967 Energy-linked ion movements in mitochondria systems. Adv Enzymol 29: 259–319
14. LEHNINGER AL, B REYNAFARJE, A VERCESI, WP TEW 1978 Transport and accumulation of calcium in mitochondria. Ann NY Acad Sci 307: 160–176
15. LYONS JM, JK RAISON 1970 Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol 45: 386–369
16. MILLER GL 1959 Protein determination for large numbers of samples. Anal Chem 31: 964
17. OZELKOK SI, RJ ROMANI 1975 Ripening and *in vitro* retention of respiratory control by avocado and pear mitochondria. Plant Physiol 36: 239–240
18. RALPH RK, SJ WOJIK 1982 Cytokinin effects on mitochondria and calcium metabolism. Plant Sci Lett 25: 321–328
19. ROMANI RJ 1978 Long-term maintenance of mitochondrial functions *in vitro* and the course of cyanide insensitive respiration. *In* G Ducet, C Lance, eds, Plant Mitochondria. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 3–10
20. ROMANI RJ, PH HOWARD 1982 The restoration of mitochondrial membrane potential, oxidative phosphorylation and respiratory control, *in vitro*. Biochem Int 4: 369–375
21. ROMANI RJ, SE TUSKES, S OZELKOK 1974 Survival of mitochondria *in vitro*. Form and function after 4 days at 20°C. Archiv Biochem Biophys 164: 743–751
22. ROUX SJ, K MCENTIRE, RD SLOCUM, TE CEDEL, CC HALE 1981 Phytochrome induces photoreversible calcium fluxes in a purified mitochondrial fraction from oats. Proc Natl Acad Sci USA 78: 283–287
23. RUSSELL MJ, SB WILSON 1978 Calcium transport in plant mitochondria. *In* G Ducet, C Lance, eds, Plant Mitochondria. Elsevier/North-Holland Press, Amsterdam, pp 175–182
24. WILLIAMSON RE, CC ASHLEY 1982 Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature 296: 647–650
25. WILSON RH, RJ GRAESSER 1976 Ion transport in plant mitochondria. *In* CR Stocking, U Heber, eds, Transport in Plants III, Encyclopedia of Plant Physiology, Vol 3. Springer-Verlag, Berlin, pp 377–397
26. WOODS CM, VS POLITO, MS REID 1984 Response to chilling stress in plant cells. II. Redistribution of intracellular calcium. Protoplasma 121: 17–24