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Evidence for Ca^{++}-Calmodulin Control of Transplasmalemma Electron Transport in Carrot Cells

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Summary: Cultured carrot cells exhibit transmembrane ferricyanide reduction through a plasma membrane redox system, which may be associated with an iron reduction and uptake system in plant roots. Here we provide evidence for the inhibition of transplasma membrane ferricyanide reduction by four different Ca^{2+}-calmodulin type antagonists, calmidazolium, trifluoperazine, pimozide and fluphenazine. These compounds inhibit in low concentrations (~ 5-10 μM) in a time-dependent manner. Higher concentrations (50-100 μM) are required to inhibit transmembrane ferricyanide reduction in 10 min rather than in 30 min. The permeable calcium chelator, TMB-8, also inhibits transmembrane ferricyanide reduction in carrot cells. Since the redox system is controlled by hormones, the effects of anticalmodulin agents on hormone response may be mediated through the redox system.

Transmembrane ferricyanide reduction in cultured carrot cells has been established by Craig and Crane (1,2,3,4), in tobacco cells by Barr, Crane and Craig (5), in yeast cells by Crane et al. (6), in corn roots by Federico and Giartosio (7) and oat roots by Rubinstein et al. (11). An enzymatic process for iron reduction and uptake by plant roots, associated with a plasma membrane redox system, has been described by Römheld and Marschner (9), and Sijmons et al. (10,11). Here we provide evidence that transmembrane ferricyanide reduction by carrot cells, associated with the plasma membrane redox system of these cells, responds to Ca^{2+}-calmodulin inhibitors. This may implicate a Ca^{2+}-calmodulin type control mechanism for the plasma membrane redox system.

Materials and Methods: Carrot cells were grown in liquid suspension culture on Murashige and Skoog's medium without agar, obtained from K. C. Biological, Inc., Lenexa, Kansas 66215 (cat. no. MM-100), and supplemented with vitamins and 2,4-D. The pH of the culture medium was adjusted to 6 with NaOH. 2,4-D was obtained from the Aldrich Chemical Co. Carrot cells were generally grown in 250 ml Erlenmeyer flasks on a rotary shaker under constant illumination from room lights.

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Cells were harvested by centrifugation in an unrefrigerated International table model centrifuge at 1500 RPM for 2 min. They were rewashed 3 x with fresh sucrose-salts solution (0.1 M sucrose with 10 mM each KCl, NaCl and $CaCl_2$). After the final wash, cells were suspended in 10-30 ml sucrose-salts solution and returned to the shaker or bubbled with air until removed for individual assays.

Transmembrane ferricyanide reduction in carrot cells was measured in 2 ways: (1) by a spectrophotometric assay in an Aminco DW-2a spectrophotometer in the dual mode, measuring the difference between 420-500 nm or (2) by incubating cells with ferricyanide in a flask on the shaker, and removing aliquots for a spectrophotometric assay at 420 nm every 5 min. For method (1), a typical reaction mixture contained carrot cells (0.003 g dry wt.), 25 mM Tris-Mes, pH 7, and sucrose-salts solution to a 1.5 ml volume. The ferricyanide concentration used was in the range of 0.1-0.4 mM. A millimolar extinction coefficient of 1 was used for calculating ferricyanide reduction rates. For method (2), the reaction mixture contained 80 ml sucrose-salts solution, 10 ml 0.25 M Tris-Mes, pH 7, and 10 ml cells (about 0.3 g dry wt.). The ferricyanide concentration added was 0.5 mM. A 5 ml aliquot removed immediately after adding ferricyanide to the other reaction components served as 0 time control. Groups of 3 aliquots were removed every 5 min. thereafter for a total time of 30 min. to study transmembrane ferricyanide reduction by these cells. The tubes were stored on ice until centrifugation to remove cells before doing a spectrophotometric assay on all of the accumulated supernatants. Ferricyanide reduction rates were calculated as in Method (1).

The dry weight of cells was determined by drying cells in a 100°C oven overnight.

Results: Figure 1 shows a spectrophotometric tracing of transmembrane ferricyanide reduction by carrot cells without membranes (A,1), without additions (A,1 and B,1) and with cells in presence of 50μM trifluoperazine (A,3) and 5 and 10 μM calmidazolium (B, 2 and 3). It can be seen that 50 μM trifluoperzine gives 51% inhibition (A,3), but 10 μM calmidazolium inhibits the reaction 40% (B,3). Low concentrations of calmidazolium (5 μM) have a stimulatory effect (B,2) not seen with other calmodulin antagonists. Differences in the degree of inhibition by anti-calmodulin agents can also be seen in Table I. Greater inhibition can be obtained when ferricyanide and a calmodulin antagonist are incubated together. The slow phase of the rate generally shows more inhibition than the fast, initial phase of the rate. When the flask method is used to measure the effect of Ca^{2+}-calmodulin antagonists, inhibition increases over time (Fig. 2) with lower concentrations of the inhibitors needed. With this combined incubation trifluoperazine inhibits transmembrane ferricyanide reduction by carrot cells best, followed by calmidazolium and pimozide. These inhibitors need time and oxidizing conditions to give best effect.

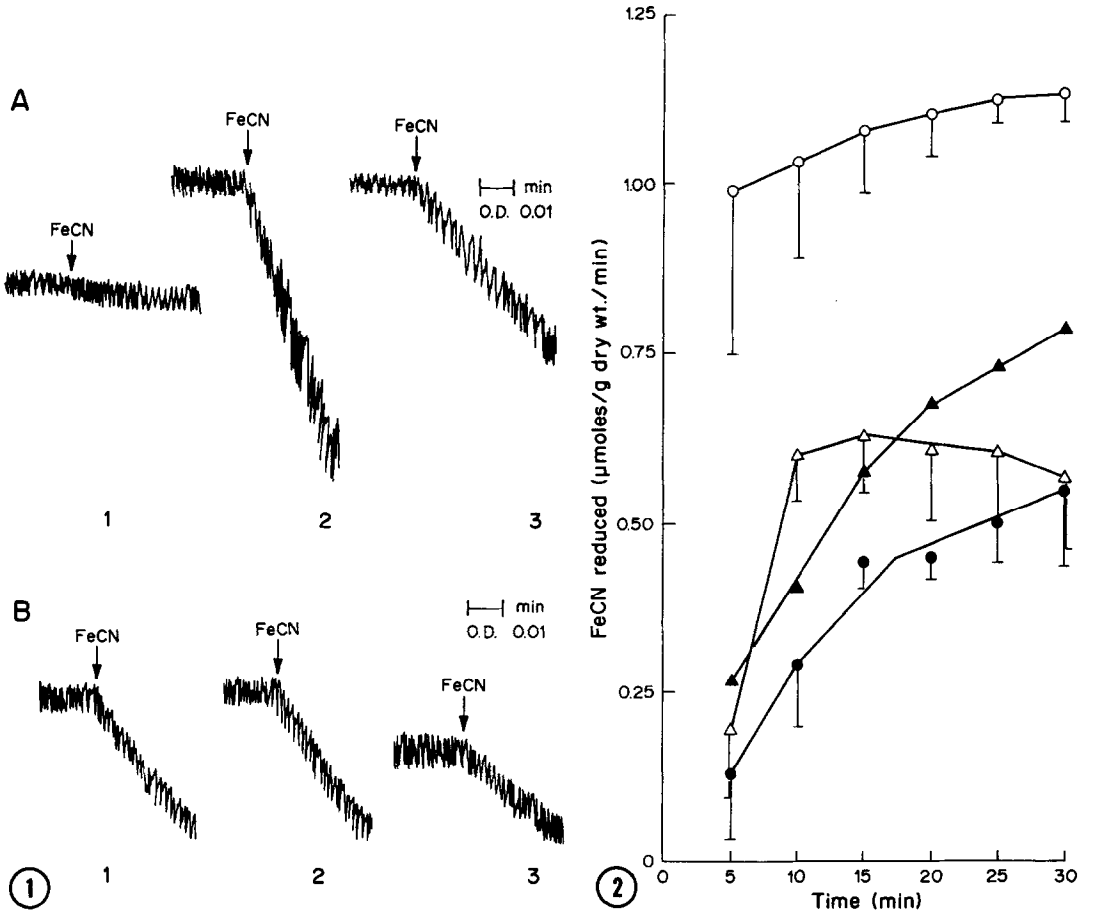

Figure 1. A Tracing of Spectrophotometric Transmembrane Ferricyanide Reduction by Carrot Cells. A,1 - no membranes; 2 - with membranes; 3 - with membranes and 50 μM trifluoperazine; B,1 - no additions; 2 - with 5 μM calmidazolium; 3 - with 10 μM calmidazolium. Reaction conditions as described in Materials and Methods for procedure (1).

Figure 2. Transmembrane Ferricyanide Reduction by Carrot Cells in Presence of Various Ca^{2+}-Calmodulin Antagonists. ○-control; ●-5 μM TFP; △-5 μM CA; ▲-5 μM PIM. Carrot cells (dry wt. about 0.03g) were incubated with 10 ml 25 mM Tris-Mes, pH 7, 0.5 mM FeCN and sucrose-salts solution to a volume of 100 ml. Calmodulin antagonists were added in concentrations indicated. Aliquots of cells for spectrophotometric FeCN reduction were removed every 5 min for a total time of 30 min.

Such Ca^{2+} antagonists as TMB-8 also inhibit transmembrane ferricyanide reduction in carrot cells (Fig. 3). Rapid penetration of TMB-8 to the active site is evident from nearly 100% inhibitions with 15 and 50 μM TMB-8 in 5 min, from which carrot cells seem to recover to a degree over 30 min.

Discussion: Transmembrane ferricyanide reduction by carrot cells is a relatively new area under investigation (1-4). It is postulated that a

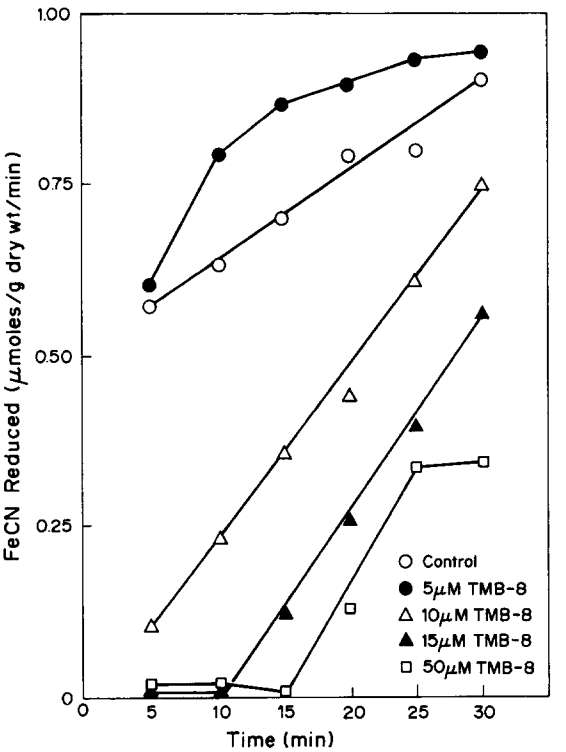

Figure 3. Inhibition of Transmembrane Ferricyanide Reduction by Carrot Cells with TMB-8, a Calcium Chelator. Reaction conditions as in Fig. 2; TMB-8 added in concentrations indicated.

reductant, such as NADH or NADPH from within the cells reduces an outside electron acceptor. The electrons liberated on the inside reach the outside of the cell via a plasma membrane electron transport chain. Ferricyanide, a non-permeable reagent, acts as an artificial terminal electon acceptor on the outside, which is easily measured. Since protons are secreted simultaneously with the reduction of ferricyanide, transmembrane ferricyanide reduction may be coupled to a proton channel or to a proton-transferring ATPase found in the plasma membrane (12).

Transmembrane ferricyanide reduction by whole cells also responds to plant hormones. As shown by Barr, Craig and Crane (5), normal tobacco callus cells are stimulated by low concentrations of IAA, gibberellic acid or benzyl adenine, while transmembrane ferricyanide reduction by transformed tobacco cells, whose growth is inhibited by the hormones, is inhibited up to 67% by similar hormone concentrations. Here we present evidence that transplasma membrane electron

Table I. A Comparison of Degrees of Inhibition of Transmembrane Ferricyanide Reduction by Carrot Cells in Presence of Calmoulin Antagonists

Antgonist	Conc.	Incubation of Cells for 10 min.			
		without FeCN but with inhibitor		with FeCN and inhibitor	
		rate[a]	inhibition	rate[a]	inhibition
	(μM)		(%)		(%)
None	-	0.80	-	0.72	-
Calmidazoliumm	20	0.54	32	0.48	33
Fluphenazine	10	0.60	25	0.54	25
Pimozide	10	0.72	10	0.60	17
Trifluoperazine	10	0.72	10	0.60	17

[a] μmoles FeCN reduced /g dry wt./min

transport in cultured carrot cells, assayed by transmembrane ferricyanide reduction, is also affected by CA^{2+}-calmodulin antagonists. As a comparison of Figures 1 and 2 with Table I shows, preincubation of carrot cells with the inhibitors for 10 min. produces greater inhibition than the addition of inhibitors to an assay with ferricyanide reduction in progress. Longer incubation periods (up to 30 min.) with ferricyanide plus various Ca^{2+}-calmodulin antagonists, such as trifluoperazine, pimozide or calmidazolium, combined, allow the concentration of inhibitors to be decreased from 50-100 μM to between 5 and 10 μM. The time factor in developing >50% inhibition of transmembrane ferricyanide reduction with low concentrations of inhibitors indicates that it is necessary for the Ca^{2+}-calmodulin antagonists to penetrate the cells to the active site and, possibly, reduce the level of available internal electron donors, NADH or NADPH, through inhibition of reactions associated with substrate and/or energy-linked reactions in the cytoplasm, which participate in normal energy production.

The low concentrations of the anticalmodulin agents which give inhibition are below concentrations commonly associated with non-specific membrane effects (13).

Many Ca^{2+}-calmodulin controlled reactions are known in the animal kingdom (14) and a few in plants, such as NAD kinase (15), a microsomal ATPase from Zea mays L.; calmodulin inhibitors can prevent the expression of a plant hormone response in Amaranthus (17,18). Since transmembrane ferricyanide reduction by carrot cells responds to Ca^{2+} chelators, such as TMB-8 (Fig. 3), it is possible that the plasma membrane redox system, measured as transmembrane ferricyanide reduction here, is another reaction controlled by a Ca^{2+}-calmodulin type regulation. Since the transplasma redox is hormone controlled, the effects of calmodulin inhibitors on growth and their effects on transmembrane redox may be related (12). Such an assumption is also partially supported by the isolation of calmodulin from barley (19), spinach (20,21), and oat roots (22) and its possible involvement in geotropic responses of roots (23).

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