

Evidence for a Trans-Plasma Membrane Electron Transport System in Plant Cells

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Introduction

Dormandy and Zarday (1) demonstrated that extracellular reduction of ferricyanide occurred with intact erythrocytes and it was proposed by Mishra and Passow (2) that this reduction occurred via a transmembrane dehydrogenase, which transferred electrons from an internal redox donor to the external ferricyanide. Orringer and Roer (3) have proposed that ascorbic acid is a redox carrier across the plasma membrane of erythrocytes and is responsible for external ferricyanide reduction. The presence of NADH-ferricyanide reductase has been established in the plasma membranes of many types of cells (4, 5) and recently extracellular ferricyanide (FeCN) reduction has been observed in yeast cells by Crane *et al.* (6), who suggest an electron transport system in the plasma membrane is responsible. In this paper we present evidence for extracellular FeCN reduction via a transmembrane electron transport system in the plasma membrane of intact carrot cells. The rate is stimulated by cations and is accompanied by an increased proton release from the cells.

Materials and Methods

A carrot cell culture line obtained from Drs. P.M. Hasegawa and R.A. Bressan of the Purdue Horticulture Department was grown in liquid suspension culture. Cells were harvested in the logarithmic phase of growth and washed with .10 M sucrose. The suspension subsequently was aerated and aliquots used as needed. Potassium ferricyanide and Na_3VO_4 were obtained from Fisher Scientific Co., sodium ferricyanide from K and K division of ICN Pharmaceuticals, Inc. Oxone, a monopersulfate oxidant, was from Dupont. Tris and Mes buffers and PCMB (p-chloromercuribenzoate) and NEM (N-ethylmaleimide) were obtained from Sigma Chemical Co., sucrose from Mallinckrodt. An Aminco DW-2a spectrophotometer was used to measure ferricyanide reduction using the dual beam mode with a reference at 500 nm to reduce problems with turbidity. The sample was stirred continuously with a magnetic stirring assembly. pH measurements were done using an Orion model 701 A pH meter with a Corning glass combination electrode. A millimolar extinction coefficient of 1.0 was used for potassium and sodium ferricyanide.

Results

Experiments were done to determine whether ferricyanide (FeCN) reduction was due to FeCN entering into cells with subsequent reduction. Cells were allowed to reduce FeCN under normal assay conditions (pH 7.0 Tris-Mes-HCl 0.05 M, 0.047 M sucrose) and total ferri-ferricyanide content was determined in the supernatant after cells were removed by centrifugation. Table I shows that nearly all of the FeCN added to the cells was reduced after 10 minutes and that an average of approximately 98% of this could be accounted for in the supernatant and not in the cells after centrifugally removing the cells. Figure 1 shows the effects of cations on FeCN reduction. CaCl_2 and MgCl_2 gave a maximum stimulation

TABLE I. Ferricyanide Recovery in Extracellular Medium Following Ferricyanide Reduction by Cells

	Ferricyanide Reduced After 10 minutes (μ Moles)	Ferricyanide Recovered in Supernatant (μ Moles)	Recovery (%)	Recovery Normalized to Sucrose Control (%)
Buffer, sucrose	.207	.195	94.2	98.8
Buffer, sucrose	.207	.197	96.7	101.4
Buffer + Cells in sucrose	.182	.174	95.6	100.2
Buffer + Cells in sucrose	.191	.178	93.2	97.7
Buffer + Cells in sucrose	.191	.173	90.6	95.0

TABLE I. Assays contained 0.05 M Tris-Mes-HCl, pH 7.0, and 0.047 M sucrose. A small amount of sodium ascorbate was added to the two buffered sucrose controls to reduce the ferricyanide for subsequent determination of recovery observed with no cells present. 7.02 mg dry wt of cells in 1.5 ml volume were allowed to reduce FeCN for 10 min., after which cells were removed by a 4 min. spin in a Beckman 152 microfuge. Oxone oxidized all ferrocyanide and the change in optical density on ascorbate addition was used for measuring total FeCN.

of the rate of 40% and 35% while KCl showed a stimulation of only 6% at the highest concentration examined. The ability of cells to reduce FeCN was unaffected by sulfhydryl reagents PCMB or N-ethyl maleimide. Orthovanadate, a plant plasma membrane ATPase inhibitor (7), had no effect on the rate of FeCN reduction in the concentration range 0.5-5.6 mM.

NADH is a possible source of intracellular reducing equivalents for external

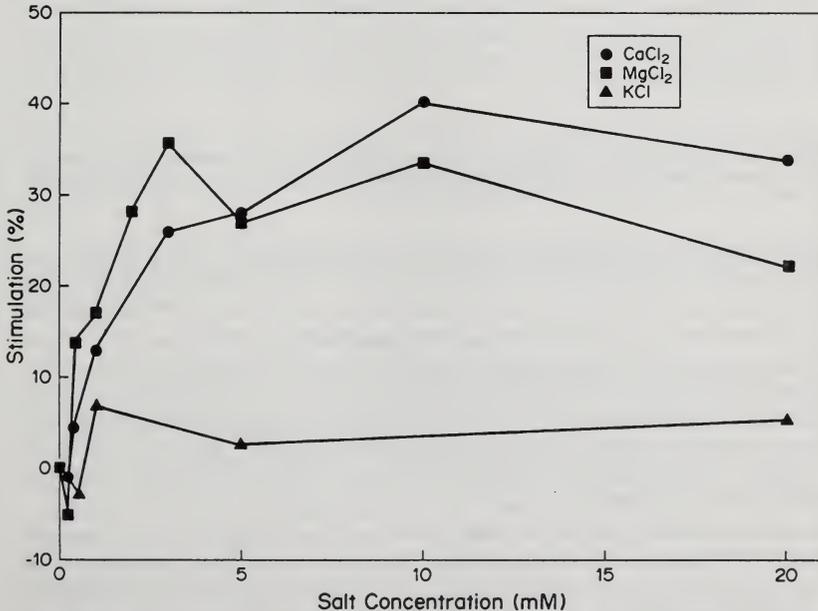


FIGURE 1. Salt Effects on Ferricyanide Reduction by Whole Cells. For KCl effects $\text{Na}_3\text{Fe}(\text{CN})_6$ (0.81mM) was added and for MgCl_2 and CaCl_2 $\text{K}_3\text{Fe}(\text{CN})_6$ (0.81mM) was added to cells in 50mM Tris-Mes-HCl, pH 7.0, and 0.047 M sucrose.

TABLE II. *Ethanol Stimulation of Starved Carrot Cells and Pyrazole Inhibition of Stimulation*

	FeCN Reduced/MIN/ MG DRY WT (NMOLE)	Effect (%)
A. 1.(-)Pyrazole	2.15	
+ 5λ Ethanol	3.29	+ 54
+ 5λ Ethanol	4.03	+ 88
2.(-)Pyrazole	2.37	
+ 10λ Ethanol	4.46	+ 88
3.(+)Pyrazole (.1 M)	2.19	
+ 10λ Ethanol	2.37	+ 8
4.(-)Pyrazole	2.42	
+ 10λ Methanol	2.25	- 7
	2.09	
+ 10λ N-Propanol	2.30	+ 10
	1.91	
+ 10λ Ethanol	3.24	+ 70
5.(+)Pyrazole (.1 M)	2.39	
+ 10λ Ethanol	2.37	- 1
B. 1.(+)Pyrazole (.1 M)	3.07	
+ 5λ Ethanol	2.66	- 13
2.(-)Pyrazole	3.99	
+ 5λ Ethanol	4.77	+ 20
3.(-)Pyrazole	1.86	
+ 5λ Ethanol	2.00	+ 8
+ 5λ	2.44	+ 31
4.(+)Pyrazole (.1 M)	1.78	
+ 5λ Ethanol	1.51	- 15
+ 5λ Ethanol	1.46	- 18

TABLE II. Cells were washed and incubated in 0.10 M Na_2HPO_4 , pH 7.0 for various lengths of time: A. 2.5 hrs. B. 1 and 2-1 hr. B. 3 and 4 - 5 hrs. Assay conditions A: 0.047 M NaPO_4 , pH 7.0 B: 0.037 M NaPO_4 , pH 7.0 A and B: 50 mM Tris-Mes- HCl, pH 7.0, 0.81 mM $\text{K}_3\text{Fe}(\text{CN})_6$.

FeCN reduction and this was investigated by using ethanol to try to increase the intracellular NADH levels in starved cells through the cytosolic alcohol dehydrogenase. Stimulation of FeCN reduction by ethanol up to 88% is observed in starved cells in Table IIA, #1 and #2 and in Table IIB, #2 and #3. Ethanol stimulation is clearly due to alcohol dehydrogenase activity, as seen by the ability of pyrazole, an alcohol dehydrogenase inhibitor, to prevent stimulation (Table IIA, #3 and #5, Table IIB, #1 and #4). Also methanol, a non-substrate, and n-propanol, a poorer substrate than ethanol for alcohol dehydrogenase fail to significantly stimulate, while subsequent ethanol addition does stimulate (Table IIA, #4) external ferricyanide reduction.

The possibility that a reducing substance comes across the membrane to reduce FeCN externally was tested by measuring the ability of the supernatant from incubated cells to reduce FeCN. Table III shows that the supernatant had only 8-9% of the activity of whole cells. Some of the remaining activity might be due to the few remaining cells in the supernatant.

Carrot cells exhibited H^+ release on addition of FeCN, as measured on a pH

TABLE III. *Ferricyanide Reducing Activity in the Supernatant of Washed Carrot Cells*

FeCN Reduced After 10 minutes in Presence of Cells (μ Moles)	Average of Whole Cell Rate (μ Moles)	FeCN Reduced After 10 minutes by Supernatant from a Suspension of Cells Incubated Without FeCN for 10 minutes (μ Moles)	% of Whole Cell Rate
.182		-	-
.191	.188	-	-
.191		-	-
-		.017	9.0
-		.015	8.0

TABLE III. 0.12 mM FeCN was added either directly to a cell suspension or to the supernatant derived from a cell suspension incubated 10 minutes. Assay conditions were 50 mM Tris-Mes-HCl, pH 7.0, 0.047 M sucrose with 7.02 mg dry wt cells per 1.5 ml cuvette volume. After incubation for 10 minutes, cells were removed by centrifugation and the supernatant was assayed for ability to reduce FeCN. Incubation took place in the presence of ^a0.3 mM KCl or ^b1.20 mM KCl.

TABLE IV. *Comparison of Ferricyanide Reduction and H⁺ Release on Ferricyanide Addition to Carrot Cells*

Rate Before FeCN Addition	nmoles H ⁺ /min/mg dry wt Rate After FeCN Addition	Net Increase in Rate of H ⁺ Release on FeCN Addition	nmoles/min/mg dry wt Rate of FeCN Reduction
23.3	31.6	8.3	1.8
31.1	39.6	8.5	2.5

TABLE IV. Tris-Mes-HCl, 1.0 mM, KCl 20 mM, K₃Fe(CN)₆ 0.81 mM. The pH was 7.3 at the start and 6.5 at the end of a run. FeCN was added to the pH or spectrophotometer cuvette 2.5 min. after cells were added to the buffer in the pH chamber (from which an aliquot of 1.5 ml was removed to measure FeCN reduction).

meter at the same time that FeCN was reduced, as shown in Table IV. Cells exhibited a rate of H⁺ release without FeCN which was stimulated on addition of FeCN.

Table V shows that 46% of the FeCN rate was dependent on O₂ presence. Addition to O₂ to deoxygenated cuvettes clearly shows that O₂ is required for maximum activity.

Discussion and Conclusions

The evidence which has been presented suggests that FeCN is not taken up by cells and lack of ability to bring about FeCN reduction in cell suspension supernatant (Table III) suggests, although it does not unequivocally demonstrate, that it is not the release of reducing compounds from the cell which is responsible for the observed rate of FeCN reduction. The lack of inhibition by sulfhydryl reagents PCMB and NEM indicates that FeCN reduction is not due to oxidation of the sulfhydryl group of a transmembrane carrier sulfhydryl compound crossing the membrane, and that it is not non-specific oxidation of membrane protein sulfhydryl groups.

The presence of a transmembrane electron transport system is an explanation for the FeCN reduction observed with whole carrot cells. Orringer and Roer (3) have argued that external FeCN reduction with human erythrocytes occurs as

TABLE V. O_2 Involvement in Ferricyanide Reduction in Carrot Cells

	nmoles FeCN Reduced/ min/mg dry wt	Average	%Change
$K_3Fe(CN)_6(4.04mM)$			
air	3.50		
air	3.43	air	3.47
+ aeration with O_2 -20 sec.	3.84		
argon	2.02	argon	1.89
+ aeration with O_2 -20 sec.	3.33		- 45.5
argon	1.75	argon	3.49
+ aeration with O_2 -20 sec.	3.64	+ O_2	

TABLE V. Cells in 50 mM Tris-Mes-HCl, pH 7.0, and 0.047 M sucrose were either aerated or had argon bubbled through them for 2.5 min and then were covered, opened momentarily for FeCN addition. In certain cases pure O_2 was bubbled through for 20 sec.

a result of NADH oxidation within the cell. Ethanol stimulation using alcohol dehydrogenase reported here suggests NADH is involved in supplying reducing equivalents on the internal face of the plasma membrane for FeCN reduction outside of carrot cells.

The findings of Mishra and Passow (2) and Dormandy and Zarday (1) support the concept of a protonophoric transmembrane NADH dehydrogenase in erythrocytes. They showed that reduction of FeCN by intact erythrocytes was accompanied by proton transfer across the membrane. Here proton release stimulated by FeCN may be due to proton translocation across the membrane as a result of electron flow from a compound, such as NADH, to FeCN outside the cell. The non-stoichiometric nature of the proton release might be due to release from control of an electron transfer pathway pumping protons with another acceptor, possibly O_2 , as the natural acceptor. Another possibility might be an effect on the plasma membrane H^+ -ATPase, stimulating it to pump protons external to the cell. Crane *et al.* (6) observed proton release, stoichiometric to external FeCN reduction in yeast cells, but added KCN to inhibit proton movement due to the plasma membrane ATPase before FeCN was added, which was not done here.

The data showing O_2 involvement in at least a part of the rate of reduction of FeCN is evidence that O_2 could be a natural electron acceptor. Superoxide could be the immediate product of such an electron transfer system and this would be able to reduce FeCN. Such a system could be envisioned to result in superoxide dismutation to H_2O_2 , which could be used in lignin biosynthesis.

The stimulations by Ca^{+2} and Mg^{+2} are very similar both in extent and saturating concentrations to those seen by Crane *et al.* (6) with yeast external FeCN reduction. They saw approximately 40% stimulation, saturating at about 5 mM, while we see here about 40% stimulation at 10 mM and about 30% stimulation at about 2.5 mM for $CaCl_2$ and $MgCl_2$, respectively. The effect of Ca^{+2} could be explained as an increase in H^+ movement, resulting in an increased electron transport rate. Ca^{+2} uptake has been shown to be coupled to proton transport in yeast (8, 9).

There are evident similarities between this electron transport system in the carrot cell plasma membrane and the ferricyanide reduction system in yeast plasma membrane and the transmembrane NADH-ferricyanide reductase of erythrocytes. In each FeCN reduction occurs and is accompanied by the transport

of protons. In each as well the rate of FeCN reduction is stimulated by calcium and magnesium ions. There may be very real similarities in the organization of the electron transport systems in the plasma membranes of these three types of cells. Such a system may be present in the plasma membranes of a diversity of organisms.

Since the NADH dehydrogenase in erythrocytes and the other animal cell membranes is responsive to hormones and it has been proposed that this dehydrogenase is involved in control of cell function (10, 11), this system may be affected by plant hormones and could be a key mediator for one or more plant hormones. Since the FeCN reduction rates are expressed on a per mg dry weight basis, NADH-FeCN reductase activity per mg plasma membrane protein would be considerably higher and more in line with values seen for NADH-FeCN reductase in isolated mammalian membranes.

Acknowledgements

This study was supported by N.I.H. Grant #AM25235.

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