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Transmembrane Ferricyanide Reduction in Tobacco Callus Cells

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Abstract. Transmembrane ferricyanide reduction in whole cells of normal and of transformed tobacco (*Nicotiana tabacum*) callus tissue was compared. It was found that low concentrations of indoleacetic acid (IAA, 0.1 μM), gibberellic acid (GA, 0.3 μM), and benzyl adenine (BA, 0.03 μM) stimulate external ferricyanide reduction in normal tobacco callus cells, but inhibit this reaction up to 67% in transformed cells when hormones are applied to cells 10 min prior to assay. Higher concentrations of these growth regulators (1 μM or greater) inhibit transmembrane ferricyanide reduction in both types of cells, with the exception of IAA, giving an initial stimulation of the rate (12%), followed by 24% inhibition after 2 min. The observed external ferricyanide reduction by whole tobacco callus cells may be explained on the basis of a transplasmalemma redox system, which may be associated with the iron metabolism of these cells.

A transplasma membrane redox system for reducing Fe^{3+} chelates to Fe^{2+} chelates by plant roots was first hypothesized by Chaney et al. (1972). Evidence for a transplasma membrane redox system in carrot tissue culture cells was first provided by Craig and Crane (1981). They showed that ferricyanide, a nonpenetrating electron acceptor, could be reduced by whole carrot cells with simultaneous proton release into the reaction medium. Subsequently, Crane et al. (1982) showed that the petite mutant of *Saccharomyces cerevisiae*, which shows little respiratory activity, could reduce external ferricyanide and the transmembrane redox system involved could be stimulated by such uncouplers as CCCP. Hormonal control of the transplasmalemma redox system was shown by Craig and Crane (1982) in carrot cells, using 2,4-D, which gave 20% inhibition.

In the present study, hormonal control of the transplasmalemma redox system is shown in tobacco callus cells. Normal as well as transformed cells responded to IAA, gibberellic acid, and benzyl adenine.

Materials and Methods

Tobacco callus cells—normal (XSR) and transformed crown gall tumor cells (E9) (Thomashow et al. 1980)—were grown in liquid suspension tissue culture on Murashige and Skoog's basal medium (1962) with vitamin B_1 and inositol supplements from Gibco. Normal cells also required naphthalene acetic acid (1 mg/l) and cytokinin (100 μg/l), but transformed cells did not.

Preparation of Whole Cells

Cells were harvested in the log phase by centrifugation in an International table model centrifuge at 500 × *g* for 5 min and homogenized in a Waring blender in sucrose-salts suspension medium (0.1 M sucrose with 10 mM KCl, $CaCl_2$, and NaCl) with on and immediately off bursts of energy at the 100 setting mark on a Variac for a total of six times to break up cell clusters, but not damage whole cells. The cell suspension was then filtered through ten layers of cheesecloth and a single layer of Miracloth before recentrifugation as above. To be sure that no broken cells were mixed with whole cells, the supernatant was discarded after centrifugation and a rewash done with fresh, cold sucrose-salts solution. The process of rewashing was repeated a total of three times. Finally, cells were suspended in about 50 ml of sucrose-salts solution and put on a shaker to provide aeration, while assays were run on aliquots withdrawn when needed. When ferricyanide was added to the supernatant after cell removal, only 1% ferricyanide reduction could be observed. This is a control for the involvement of whole cells in transmembrane ferricyanide reduction. To show that ferricyanide does not penetrate tobacco callus cells, 99% recovery of ferricyanide from the supernatant could be obtained after removal of cells by centrifugation and reoxidation of reduced ferricyanide with H_2O_2, as was shown for carrot cells by Craig and Crane (1981, 1982).

Spectrophotometric Assays for Ferricyanide Reduction

Ferricyanide reduction was measured on an Aminco-Chance DW-2 spectrophotometer in the dual wavelength mode at 420 nm for ferricyanide reduction minus 500 nm as the reference wavelength to correct for turbidity. The sensitivity was set at 0.1. The sample was continuously stirred with a magnetic stirring assembly. The rates were recorded with a Linear Model recorder. The assay medium (1.5 ml total) consisted of 0.63 ml sucrose-salts, 0.19 ml 0.25 M Tris-Mes, pH 7, and 0.63 ml cells. After equilibration for 3–5 min to establish a baseline, 400 μM ferricyanide was added. The assay was continued for up to 10 min. Rates were calculated, using a millimolar extinction coefficient of

Table 1. The effect of IAA on transmembrane ferricyanide reduction in normal and transformed tobacco cells.

IAA concentration (μM)	Ferricyanide reduction		
	Initial rate (nmoles/g dry wt/min)	After 2 min	No. trials[a]
	Normal cells		
Control	548 ± 108	285 ± 45	3
0.1	886 ± 118[b]	301 ± 28	4
0.3	798 ± 306	285 ± 35	5
1.0	612 ± 79	211 ± 30[c]	3
	Transformed cells		
Control	526 ± 53	141 ± 25	4
0.01	230 ± 32[b]	77 ± 13[b]	4
0.1	176 ± 32[b]	62 ± 5[b]	4
1.0	172 ± 54[b]	82 ± 13[b]	4

[a] Trials refer to the number of assays performed, shown with standard deviations.
[b] Significant at $p = 0.05$.
[c] Significant at $p = 0.01$.

1 for ferricyanide. The dry weight of cells was obtained by drying 0.63 ml cells overnight at 100°C.

Hormone Effects on Ferricyanide Reduction

To study hormone effects on transmembrane ferricyanide reduction, the tobacco callus cells were incubated in the reaction cuvette with various concentrations of IAA, gibberellin, or 6-benzylaminopurine (benzyl adenine) for 10 min with stirring on a magnetic stirrer at room temperature. In the last 4 min of incubation, the reaction cuvette was transferred to the stirring assembly in the DW-2 spectrophotometer and a baseline for these cells was recorded before the addition of ferricyanide at the 10 min mark.

Results

In Tables 1–3 data are presented for transmembrane ferricyanide reduction in normal versus transformed tobacco callus cells. There is only a slight difference (up to 22%) in the initial rates between the two types of cells. The ferricyanide reduction rate after two min by the transformed cells is at least 36% less than that of the normal cells ($p = 0.01$). The response of the normal versus transformed cells to the various growth regulators used in this experiment differ significantly. IAA (0.1 μM) stimulates the initial rate of transmembrane ferricyanide reduction in normal cells up to 62% ($p = 0.01$), while the same concentration of IAA inhibits activity 66% in the transformed cells. After 2 min, the stimulation is not seen in the normal cells, but inhibition continues in the transformed tobacco cells. Low concentrations of gibberellic acid show a sim-

Table 2. The effect of GA on transmembrane ferricyanide reduction in normal and transformed tobacco callus cells.

GA concentration (μM)	Ferricyanide reduction		
	Initial rate (nmoles/g dry wt/min)	After 2 min	No. trials[a]
	Normal cells		
Control	785 ± 44	282 ± 26	3
0.3	984 ± 119[b]	272 ± 65	3
0.6	690 ± 51[b]	251 ± 0	3
0.9	732 ± 107	272 ± 30	3
3.0	774 ± 129	241 ± 15[c]	3
	Transformed cells		
Control	676 ± 37	181 ± 13	4
0.01	364 ± 95[c]	132 ± 31[b]	4
0.03	422 ± 78[c]	189 ± 33	4
0.1	410 ± 36[c]	132 ± 36[b]	4
1.0	288 ± 57[c]	127 ± 28[c]	4

[a] Trials refer to the number of assays performed, shown with standard deviations.
[b] Significant at $p = 0.05$.
[c] Significant at $p = 0.01$.

ilar effect to IAA-stimulation of ferricyanide reduction in normal, but inhibition in transformed cells. Benzyl adenine in low concentrations (0.03 μM) also stimulates transmembrane ferricyanide reduction in normal tobacco callus cells after a 10-min incubation, but it does not inhibit the rate as severely as IAA in transformed cells, in which inhibition exceeds 30%.

Discussion

Plasma membrane redox systems, to which the auxin effects seen here may be related, are better established in animal than in plant cells. Following early observations by several authors that ferricyanide, an anionic species carrying three negative charges, could not penetrate cells but induced ATP synthesis inside whole erythrocytes, Mishra and Passow (1969) showed that ferricyanide was reduced on the outside by a transmembrane dehydrogenase, which transferred electrons from an internal redox donor to the external ferricyanide. Orringer and Roer (1979) then noted that dehydroascorbate enhanced ferricyanide reduction by erythrocytes and proposed that dehydroascorbate could be involved in a transmembrane redox system, acting as a redox carrier to reduce ferricyanide outside of the cell. Later it was shown (MacKellar et al. 1979, Löw et al. 1980) that open ghosts have more NADH:ferricyanide activity than do closed vesicles. A NADH:ferricyanide reductase has been purified from erythrocytes by Wang and Alaupovic (1978). Clark et al. (1981) have shown extracellular ferricyanide reduction by perfused rat liver and isolated hepatocytes.

The involvement of hormones in the regulation of the transplasma membrane

Table 3. The effect of benzyl adenine on transmembrane ferricyanide reduction in normal and transformed tobacco callus cells.

BA concentration (μM)	Ferricyanide reduction		
	Initial rate (nmoles/g dry wt/min)	After 2 min	No. trials[a]
	Normal cells		
Control	676 ± 37	286 ± 85	3
0.01	573 ± 101	237 ± 0	3
0.03	632 ± 28[b]	218 ± 45[b]	3
0.1	869 ± 220	237 ± 0	3
0.3	791 ± 74[b]	237 ± 0	3
1.0	774 ± 50[b]	225 ± 20[c]	3
	Transformed cells		
Control	526 ± 53	149 ± 22	4
0.01	672 ± 19[c]	119 ± 24	3
0.03	431 ± 66[b]	91 ± 30[c]	4
0.1	459 ± 68	89 ± 25[b]	3
0.3	234 ± 39[c]	99 ± 14[c]	3
1.0	231 ± 10[c]	95 ± 15[c]	4
3.0	221 ± 21[c]	103 ± 17[c]	6

[a] Trials refer to the number of assays performed, shown with standard deviations.
[b] Significant at $p = 0.05$.
[c] Significant at $p = 0.01$.

redox system in animal cells is also established. Clark et al. (1982) found that glucagon inhibited the external ferricyanide reduction 30% at a concentration of 0.1 nM, while ritodrine, a β-agonist, inhibited ferricyanide reduction but had no effect on glucose release by perfused rat liver.

The initial rates of transmembrane ferricyanide reduction by tobacco callus cells are higher than rates after 2 min (Fig. 1). This phenomenon has also been observed with yeast cells (Crane et al. 1982), in perfused heart and rat liver (Clark et al. 1981, 1982) and other cells tested to date. It is postulated that the slower rate may result from the depletion of a substrate in these cells; and it is known that ferricyanide inhibits an IAA-dependent increase in O_2 uptake in sections of maize coleoptiles, as shown by Polevoy and Salamatova (1977).

The stimulation or inhibition of external ferricyanide reduction by IAA, GA, or benzyl adenine in tobacco callus cells shown here may belong to a broader category of hormone-induced effects for growth regulation in plants. In addition, the transplasma membrane ferricyanide reduction may be associated with iron uptake by plant cells. Chaney et al. (1972) showed that Fe^{III} chelates had to be reduced to Fe^{II} prior to uptake by soybeans. Bienfait et al. (1982) found evidence for an enzymatic reduction of iron, i.e. the presence of a reductase, which would mediate transmembrane electron transfer. The process of iron uptake is accelerated under conditions of iron stress, and it does not depend on the release of phenolics as reductants (Römheld and Marschner 1983). Sijmons and Bienfait (1983) present strong evidence that cytosolic NADPH is the source of electrons for the reduction of ferric chelates to the ferrous form

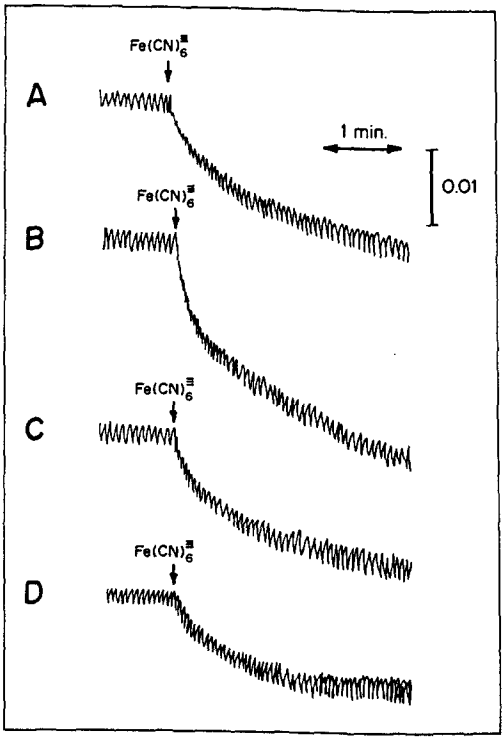

Fig. 1. Spectrophotometric tracings of transmembrane ferricyanide reduction in tobacco callus cells (420–500 nm). A: control in untreated normal cells; B: normal cells treated with 0.1 μM IAA; C: control in crown gall transformed cells; D: transformed cells treated with 0.1 μM IAA.

before uptake by iron-deficient bean roots. Such a process definitely involves a transplasma membrane redox system.

At present it is not known whether this transmembrane redox system is the same as the transplasma membrane ferricyanide reduction studied here. It would be of interest to determine if the iron chelate reduction by roots responds to hormones as the tobacco callus cells. Further studies are in progress to define the parameters of external ferricyanide reduction in normal versus transformed tobacco cells and the mechanism involved in their response to growth regulators.

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