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Depolarization of Cell Membrane Potential during Trans-Plasma Membrane Electron Transfer to Extracellular Electron Acceptors in Iron-Deficient Roots of *Phaseolus vulgaris* L.[1]

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ABSTRACT

Transfer of electrons from the cytosol of bean (*Phaseolus vulgaris* L.) root cells to extracellular acceptors such as ferricyanide and $Fe^{III}EDTA$ causes a rapid depolarization of the membrane potential. This effect is most pronounced (30–40 millivolts) with root cells of Fe-deficient plants, which have an increased capacity to reduce extracellular ferric salts. Ferrocyanide has no effect. In the state of ferricyanide reduction, H^+ ($1H^+/2$ electrons) and K^+ ions are excreted. The reduction of extracellular ferric salts by roots of Fe-deficient bean plants is driven by cellular NADPH (Sijmons, van den Briel, Bienfait 1984 Plant Physiol 75: 219–221). From this and from the membrane potential depolarization, we conclude that trans-plasma membrane electron transfer from NADPH is the primary process in the reduction of extracellular ferric salts.

Iron-deficient dicotyledonous plants develop a high capacity to reduce ferric chelates at the root surface (7, 8), a process that is part of the uptake mechanism for iron (10). We proposed recently, both on the basis of the kinetics of ferric reduction (5, 6) and the high potential supply of the cytosolic electron donor (24, 25), that iron-deficient roots reduce ferric salts by a plasmalemma-bound enzyme system which transfers electrons from cytosolic NADPH to extracellular Fe^{III}. The possibility of trans-plasma membrane electron transfer was already suggested by Chaney *et al.* in 1972. Indications for such a process were found both with iron-sufficient plant cells (11, 13, 15) and with iron-deficient roots (3, 23, 24). Under steady-state conditions, the charge carried by such a transfer of electrons should be balanced by an opposite charge transfer carried by electrogenic ion transport, *e.g.* H^+ extrusion. However, if initially electron transport and the balancing ion transport are out of phase, the addition of reduceable substrate should induce a sudden change in the membrane potential. This change may be a hyperpolarization if electron transport lags behind, or a depolarization when electron transport is the primary process. In this paper, we report the effect of extracellular electron acceptors on the membrane potential of iron-deficient and control root cells. Micro-electrode measurements demonstrate a rapid membrane depolarization of iron-deficient young root cells in the presence of extracellular electron acceptors which indicates a trans-plasma membrane electron transport system.

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MATERIALS AND METHODS

Growth of Plants. Iron-deficient and control bean plants (*Phaseolus vulgaris* L. cv Prélude, from Sluis, Enkhuizen, The Netherlands) were grown on Knop nutrient solution as described elsewhere (25) without or with 40 μM Fe-Na-EDTA as an iron source.

Ferricyanide Reduction. Extracellular ferricyanide reduction was measured in 63 ml Knop nutrient solution + 5 mM Mes (pH 4.5), and varying amounts of $K_3Fe(CN)_6$, at 25°C. The rate of reduction was measured as the decrease in A_{420} after correction for A_{500}. The pH-dependence of ferricyanide reduction was determined in 63 ml 10 mM (Tris + Mes), 0.5 mM $Ca(NO_3)_2$, and 0.2 mM $K_3Fe(CN)_6$, at 25°C.

Cell Membrane Potential Measurements. Freshly cut root tips of about 30 mm length were used. Membrane potentials of cortical cells were measured after insertion of microelectrodes at about 10 mm from the root tip, behind the elongation zone. Measuring electrodes were of the normal AgAgCl type filled with 3 M KCl brought at pH 2 with HCl. The resistance of the intracellular electrodes was about 10 MΩ and the tip potential never exceeded 10 mv. Readings were not corrected for this tip potential. Experiments were performed in 0.1 strength Knop solution to which 10 mM glucose was added (14, 16). When the potassium salts of ferricyanide (0.5 mM) or ferrocyanide (0.38 mM) were tested, KCl was added to the control (indicated by KCl in the figures) to match the total K^+ concentrations. The same was done in the case of Fe-Na-EDTA (0.2 mM) with NaCl.

Net Apparent H^+ Efflux Measurements. Plants were transfered with their roots to a solution of 0.5 mM $CaSO_4$, and after 15 min to 60 ml 0.5 mM $CaSO_4$ which was bubbled through with CO_2-free air. The pH was kept constant at 5.3 by continuous titration with 2 mM KOH. The reaction was started by addition of $K_3Fe(CN)_6$ to give a final concentration of 0.5 mM. The reduction of ferricyanide was followed spectrophotometrically at 420 nm. In one experiment, H^+ extrusion was measured in a medium such as used for the K^+ efflux measurements, with replacement of Mes-Na^+ by NaCl. The experiments were performed in plastic containers.

K^+ Efflux Measurements. Plants were transferred with their root systems to 60 ml of a medium consisting of 0.5 mM $CaSO_4$, 5 mM Mes-Na^+, 60 μM KCl, plus 1.5 mM NaCl at pH 5.3; after 2 h this medium was renewed and K^+ efflux was measured for 2

h (preincubation). Then the medium was replaced by 0.5 mM $CaSO_4$, 5 mM Mes-Na^+, 60 μM KCl, plus 0.5 mM $Na_3Fe(CN)_6$ or, in the control, 1.5 mM NaCl at pH 5.3. K^+ efflux was determined by flame spectrophotometry on samples taken every 15 min. Ferricyanide reduction was measured spectrophotometrically.

RESULTS

Extracellular Ferricyanide Reduction. Iron-deficient roots reduced ferricyanide at increased rates (8.4 ± 2.8 μmol·h^{-1}·g FW^{-1}, $n = 9$) compared to control roots (2.8 ± 1.2 μmol·h^{-1}·g FW^{-1}, $n = 5$). This increase in reduction activity corresponded with the increase that is measured with Fe^{III}EDTA as a substrate (23). The Lineweaver-Burk plots obtained with both substrates were comparable (Fig. 1); they show a linear dependence on substrate concentration at lower values, indicating the influence of diffusion by the substrate to the reaction site (6, and *cf.* 9). The pH dependency curve for ferricyanide reduction is shown in Figure 2.

Effect of Fe^{III}-Salts on the Cell Membrane Potential. Preliminary experiments showed the membrane potential in a complete Knop nutrient solution to be 30 to 40 mv more positive than in a 0.1 strength nutrient solution. At the higher concentration, the membrane potential was dominated by the diffusion potential and it was less sensitive to anoxia. Therefore, 0.1 strength Knop solution was used, in which any change in the electrogenic component of the membrane potential should be more evident. Glucose at 10 mM was added to the solution in order to avoid the risk that energy metabolism was inhibited by lack of sugars with a concomitant effect on the membrane potential (14, 16). The membrane potential of freshly cut root segments was generally rather low (−60 mv), but hyperpolarized to −120 to −130 mv within 1 to 2 h after cutting. Such a wounding effect has been observed by others (2). After reaching this stable, hyperpolarized level, the experiment proper was started. Figure 3 shows the effect of Fe^{III} and Fe^{II} given as ferri- and ferro-cyanide, respectively, on an iron-deficient and control plant. Ferrocyanide had only a minor effect, a depolarization of + 2 mv, or no effect at all. Ferricyanide on the contrary had a strong depolarizing effect on the membrane potential of the iron-deficient root cells, and a weaker, but significant, depolarizing effect on the control (Fig. 3). The depolarization was reversible. The procedure could be repeated several times but the depolarization caused by ferricyanide tended to become smaller. Fe^{III} given as Fe-Na-EDTA (Fig. 4) and DCPIP[2] in its oxidized form (not shown) caused a similar depolarization of the membrane potential.

During ferricyanide reduction at pH 5.3, there was a net H^+ extrusion, at a rate of 1 H^+/2 ferricyanide reduced (see Table I). K^+ efflux was stimulated by ferricyanide reduction. After correction for the efflux in parallel experiments (nos. 5 and 6, Table I) as a percentage of the K^+ efflux in the preincubation, the ratios

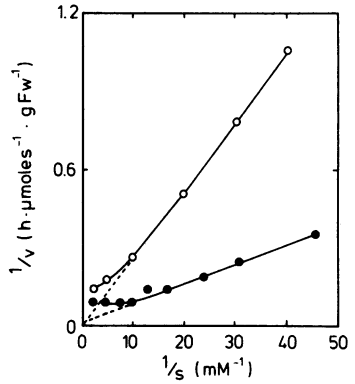

FIG. 1. Lineweaver-Burk plot for the reduction of extracellular Fe-EDTA (O) and $K_3Fe(CN)_6$ (●) by roots of iron-deficient bean plants. The data for Fe-EDTA reduction are taken from Bienfait *et al.* 1983 (6).

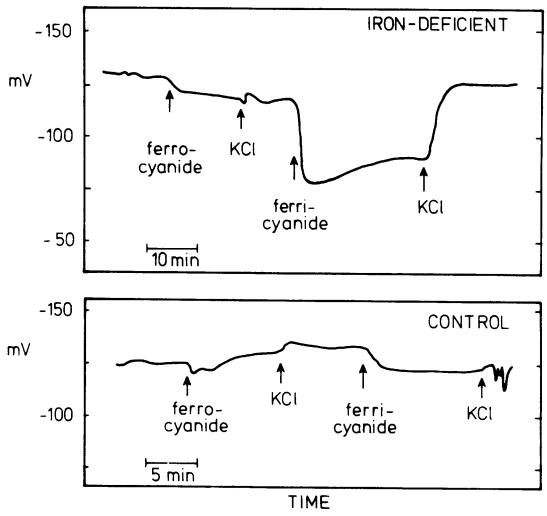

FIG. 3. Effect of ferricyanide on membrane potential of iron-deficient and control root cells. Ferro- and ferricyanide were added to the bathing solution at final concentrations of 0.38 and 0.5 mM, respectively. KCl indicates the replacement of $K_3Fe(CN)_6$ by KCl to match the total K^+ concentration.

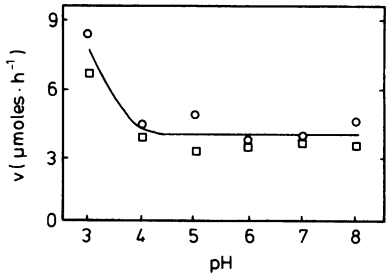

FIG. 2. pH dependence of ferricyanide reduction by roots of iron-deficient bean plants. Two plants were used for the measurements, one (O) in the order of pH 5-8-7-6-4-3-5, one (□) in the order pH 5-8-7-6-4-3-5. One measurement at each pH was 15 min, a decrease in activity between the first and the last measurements at pH 5 was 15% and 6%, respectively, and was corrected for. Reduction rates are expressed per intact root system.

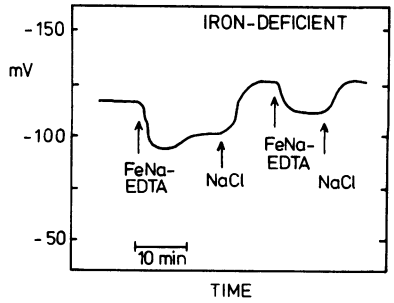

FIG. 4. Effect of FeNaEDTA on the membrane potential of iron-deficient root cells. FeNaEDTA was added to a final concentration of 0.2 mM. NaCl indicates replacement of FeNaEDTA by NaCl to match the total Na^+ concentration.

[2] Abbreviations: DCPIP, dichlorophenolindophenol; DCCD, *N,N'*-dicyclohexylcarbodiimide; FW, fresh weight.

Table I. *H^+ and K^+ Efflux during Reduction of Ferricyanide by Intact Roots of Iron-Deficient Bean Plants*

The rates of reduction and efflux are expressed as $\mu mol \cdot h^{-1} \cdot g\ FW^{-1}$

Exp. No.	Basic Incubation Medium	Preincubation		Incubation			
		Added in preincubation	K^+ efflux	Added in incubation	$Fe(CN)_6$ reduced	H^+ efflux	K^+ efflux
1	0.5 mM $CaSO_4$		ND[a]	0.5 mM, $K_3Fe(CN)_6$	10.1	5.0	ND
2	0.5 mM $CaSO_4$		ND	0.5 mM, $K_3Fe(CN)_6$	5.3	3.0	ND
3	0.5 mM $CaSO_4$		ND	0.5 mM, $K_3Fe(CN)_6$	9.5	5.7	ND
4	0.5 mM $CaSO_4$, 60 μM KCl, 4.5 mM NaCl	1.5 mM NaCl	ND	0.5 mM, $Na_3Fe(CN)_6$	9.3	4.7	ND
5	0.5 mM $CaSO_4$, 60 μM KCl, 5 mM Mes-Na	1.5 mM NaCl	1.3	1.5 mM NaCl		ND	1.2
6	0.5 mM $CaSO_4$, 60 μM KCl, 5 mM Mes-Na	1.5 mM NaCl	1.5	1.5 mM NaCl		ND	0.9
7	0.5 mM $CaSO_4$, 60 μM KCl, 5 mM Mes-Na	1.5 mM NaCl	1.1	0.5 mM, $Na_3Fe(CN)_6$	2.4	ND	2.6
8	0.5 mM $CaSO_4$, 60 μM KCl, 5 mM Mes-Na	1.5 mM NaCl	0.7	0.5 mM, $Na_3Fe(CN)_6$	4.7	ND	5.4

[a] Not determined.

of K^+/ferricyanide reduced were 0.7 and 1.0 in experiments 7 and 8, respectively (Table I). During control incubations, without ferricyanide and Mes, H^+ extrusion was lower than 0.1 $\mu mol \cdot h^{-1} \cdot g\ FW^{-1}$; in experiments 5 to 8 (Table I), Mes was added in order to stabilize pH.

DISCUSSION

The high reduction capacity at the roots of iron-deficient bean plants has a very low selectivity with regard to the electron acceptor: ferric chelates of organic acids (1, 6, 10, 22), but also DCPIP, phenazinemethosulfate (24), and ferricyanide (8) are easily reduced. The highest rates were obtained with ferricyanide.

Ferricyanide or ferrocyanide are not taken up at appreciable rates by the roots. The uptake of Fe^{III} ions, resulting from the reduction of $Fe^{III}EDTA$, is under the conditions used at least 20 times slower than the reduction of Fe^{III} (*cf.* 10 and Bienfait, unpublished). We may therefore assume that short incubations of root tissue with ferricyanide or $Fe^{III}EDTA$ do not significantly change the iron status of the cells, at least not on the time scale of the experiments reported in this communication. Consequently, any short-term effects on the metabolism should be via the extrusion of reducing equivalents.

The flat pH dependency curve for ferricyanide reduction between pH 4 and 8, in contrast to the curves found with Fe-EDTA at high (3, 5, 23) or low (6) concentrations, make this substrate ideally suited for measurements of H^+ and other ion movements during its reduction.

It was demonstrated earlier (25) that the addition of ferricyanide to iron-deficient roots results in a rapid decrease of the cellular $NADPH/NADP^+$ ratio, whereas the ferricyanide effect on the redox state of pyridine nucleotides in control roots was not significant. The rapid reversible depolarization upon addition of ferric salts, as shown in this paper, was also most pronounced with iron-deficient roots. This correlated response of NADP redox state and membrane depolarization to ferricyanide addition is direct evidence for the hypothesis that electrons are transferred across the plasma membrane during extracellular Fe^{III} reduction.

The depolarization upon incipient ferric reduction does not necessarily mean that only electrons are exported during the initial phase, but merely that there is an imbalance in the transport of charges. If initially electrons and protons are transported across the plasma membrane in a ratio of 2 e/H^+ (as indicated by the results in Table I) without a charge-balancing transfer of other ions, a depolarization should result. The depolarization was completed within 3 min after addition of ferricyanide, and often followed by a slight repolarization. Reduction of Fe^{III} salts continues at a constant rate for a far longer time (6); this means that after 3 min electron (+ proton) transfer is charge-balanced by transport of ions other than H^+.

A plausible candidate for this charge-balancing ion is K^+. Indeed, reduction of Fe^{III} is accompanied not only by an efflux of H^+ but also of K^+. The total charge carried by the efflux of K^+ and H^+ exceeds that of electron transport (Table I); thus, transport of other ion species, *e.g.* Na^+ influx or an anion efflux, must also be involved.

It must be emphasized that the proton extrusion that is evoked by the addition of extracellular Fe^{III}, as described here, is distinctly different from the proton release during the so-called acidification cycle of iron-deficient roots (4, 7). The latter type of proton extrusion is correlated with the induction of rhizodermal transfer cells (22) and occurs in the absence of any reduceable substrate in the nutrient solution (4).

Lin, working with corn protoplasts, reported no net export of protons in a system which oxidized extracellular NADH in the presence of DCCD (17). As oxidation of NADH consumes 1 $H^+/2$ electrons, the cells of Lin should have exported at least that proton per NADH oxidized, in a DCCD-insensitive way, *i.e.* directly coupled to the transfer of 2 electrons. A direct comparison between the extracellular NADH oxidase and ferricyanide reductase activities would be useful in this respect.

Crane *et al.* reported a stoichiometric export of electrons and protons during ferricyanide reduction by yeast cells (12). With cultured carrot cells, however, Craig and Crane (11) observed a proton export which was 3-fold the reduction rate of ferricyanide. In their carrot cell experiment, the KCl concentration (20 mM) was much higher than in the yeast experiment (not more than 3 mM) so that a secondary effect via an exchange of H^+ against K^+ may have complicated the picture with the carrot cells.

In the past, many models have been proposed in which transport of electrons or reducing equivalents in the plasma membrane were supposed to be the primary process in ion transport in higher plant cells (18, 21). In bacteria and other microorganisms, electron transport chains coupled to H^+ transport, together with proton pumping ATPases, are still considered to be the driving force for ion transport. In higher plant cells, however, ATP, via proton pumping ATPases, has been considered more and more the exclusive energy source for ion transport across the plasma membrane (20). But recently, it has been proposed that reducing equivalents from NADH could directly drive elec-

trogenic H^+ extrusion across the plasmalemma via a redox chain located there, without ATP as an obligatory intermediate source of energy (11, 17, 19, 26). Apparently there is, in the plasma membrane of higher plant cells, next to the generally accepted ATPase-driven electrogenic H^+-pump, another electrogenic mechanism: a H^+ extrusion pump fueled by electron transport from NADH or NADPH to an extracellular acceptor, such as a ferric salt (this work) or to oxygen (17, 26).

In conclusion, we propose that the primary process of extracellular Fe^{III} reduction is electron transfer across the plasma membrane coupled to proton transport in a ratio of $2e/H^+$. This mechanism is electrogenic and depolarizes the cell membrane potential. Under steady-state conditions, the process is charge-balanced by other ion fluxes, among others an efflux of K^+.

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