Redox-induced Proton Excretion by Cultured Carrot Cells Is Affected by Protonophores and Inhibitors of ATPase

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Introduction

Proton excretion by plant cells in well known. It has been attributed to the action of the H^+-ATPase, located on the cytoplasmic surface of the plasma membrane (13, 15). Recently, redox energy-driven proton pumps have also been described (7-9). The first report of transmembrane ferricyanide reduction associated with H^+ excretion, by Craig and Crane, appeared in these proceedings (2,3). In the present communication we provide evidence that the proton excretion exhibited by cultured carrot cells is derived from two different sources: 1) the action of an H^+-ATPase and, 2) from a transplasma membrane electron transport chain.

Materials and Methods

Carrot cells were grown in 250 ml Erlenmeyer flasks on a rotary shaker in 50 ml of Murashige and Skoog's (10) liquid medium without agar, obtained from K.C. Biological, Inc., Lenexa, KS, 66215. Cells were harvested between 4-8 days by centrifugation in 15 ml centrifuge tubes in a table model unrefrigerated International centrifuge for 2 min. at 1500 r.p.m. They were rewashed 3 times with a sucrose-salts solution (0.1 M sucrose with 10 mM each of KCl, $MgCl_2$ and $CaCl_2$). The final cell pellet was suspended in 50 ml of sucrose-salts solution and placed on a reciprocal shaker or supplied with a thin stream of air from a laboratory airline. Aliquots from this cell suspension were withdrawn for use in assays. These carrot cells remained in good condition for at least 8 hrs.

Transmembrane ferricyanide reduction of cultured carrot cells was measured spectrophotometrically with a DW-2A spectrophotometer as the difference between 420 and 500 nm at 24 °C, as previously described (2,3). The initial reaction mixture in 1.5 ml consisted of 25 mM Tris-Mes, pH 7, and 0.005 g cells (dry wt.), and 150 μM FeCN was added to start the reaction after a 3 min. incubation period. In some cases, cells were incubated with inhibitors for up to 30 min. Aliquots of cells were withdrawn every 10 or 15 min. to test for rates of ferricyanide reduction.

Proton excretion by cultured carrot cells was measured with a combination pH electrode (Corning) in a 5 ml water jacketed cell at 24 °C, gently stirred with a magnetic stirring bar. Additions of uncouplers and inhibitors were made through a sidearm. Air from a laboratory airline, filtered through cotton, was bubbled through the cell suspension from another sidearm of the flask. H^+ excretion was monitored with a Linear model recorder. Rates of H^+ excretion were calculated from the addition of known amounts of HCl to the reaction mixture after every assay.

If 5 μl of 0.01 N HCl were added to the reaction mixture after the assay, 0.05 μ equivalents HCl or H^+ resulted, which was equivalent to 50 squares on the chart, for example. The number of squares given by the basal rate (x), divided by 50 squares from the acid, and divided by the dry weight of cells in g, gave the rate of H^+ g dry wt.$^{-1}$ min.$^{-1}$

Results and Discussion

It has recently been acknowledged (4,7-9) that transplasma membrane electron transport can contribute to H^+ excretion by plant cells. This conclusion was based on studies by Craig and Crane (2,3) with cultured carrot cells, Federico and Giartosio (5),

Qiu, Rubinstein and Stern (11) and Böttger and Lüthen (1) with maize root segments, Rubinstein, Stern and Stout (12) with oat root segments, Thom and Maretzki (16) with sugarcane protoplasts, Sijmons *et al.* (14) with bean roots and Ivankina and Novak (6)

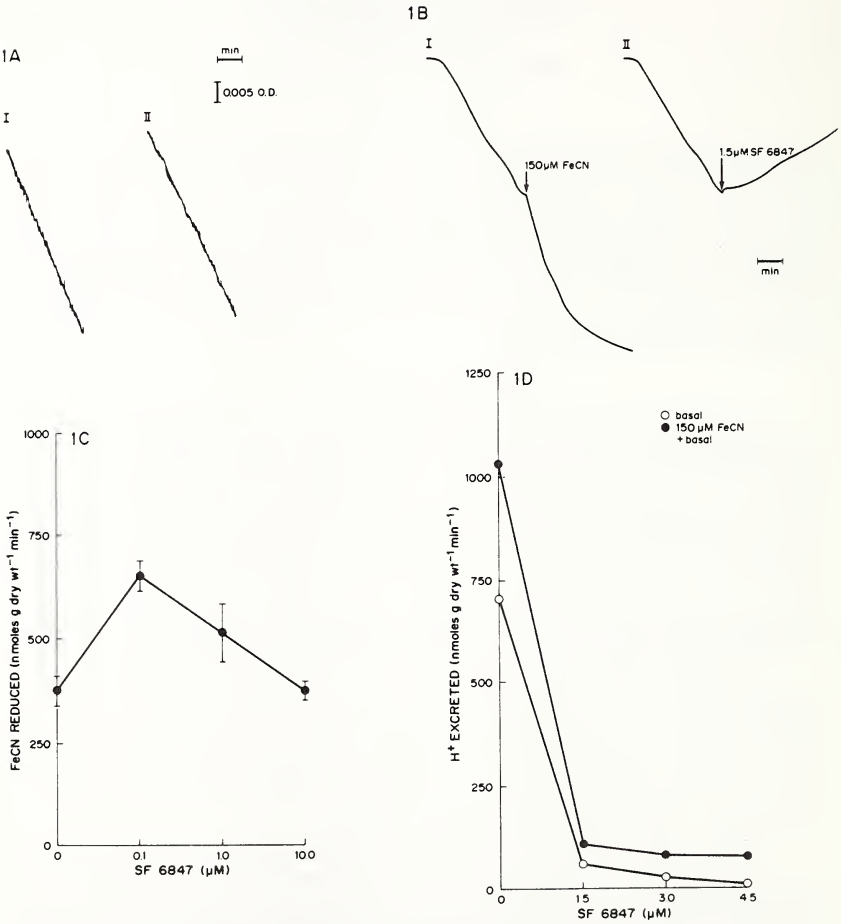

FIGURE 1A. Tracings of Transmembrane Ferricyanide Reduction by Cultured Carrot Cells. I — control rate was 385 nmoles FeCN reduced g dry wt.$^{-1}$ min^{-1}; II — rate with 10 μM SF 6847 was 350 nmoles FeCN reduced g dry wt.$^{-1}$ min^{-1}.

FIGURE 1B. Tracings of H^+ Excretion by Cultured Carrot Cells. I — control showing a basal rate of 700 nmoles H^+ excreted mg dry wt.$^{-1}$ min^{-1} before the addition of FeCN; 1050 nmoles with FeCN added. II — the rate of H^+ excretion in presence of 1.5 μM SF 6847 was 0.

FIGURE 1C. The Effect of SF 6847 on Transmembrane Ferricyanide Reduction by Cultured Carrot Cells. Various concentrations of SF 6847 were added as shown.

FIGURE 1D. The Effect of SF 6847 on H^+ Excretion by Cultured Carrot Cells. Various concentrations of SF 6847 were added as shown. Note inhibition of both basal and ferricyanide induced proton release.

with *Elodea* leaves. In the present study, we differentiate between the two sources of protons by comparing the effects of selected ATPase and mitochondria inhibitors on basal and ferricyanide-induced proton release. ATPase inhibitors are expected to inhibit the protons excreted through the action of the H^+-ATPase, and mitochondria electron transport inhibitors are expected to reduce the source of energy, ATP, needed for ATPase action. The amount of ATP available to the cell should not affect transplasma membrane electron transport, since it derives energy from the oxidation of intracellular NADH by the external electron acceptor.

The basic methods of data collection are illustrated in Figure 1, where a tracing of transmembrane ferricyanide reduction (A) portrays plasma membrane electron transport and a tracing of the lowering of pH in the reaction medium (B) illustrates H^+ excretion by carrot cells. Uncouplers, such as SF 6847, show more effect on H^+ excretion (Figure 1D) than on ferricyanide reduction (Figure 1C). The best known ATPase inhibitors, diethylstilbestrol and DCCD, inhibit the basal or ATPase-generated H^+ excretion, while ferricyanide reduction by carrot cells was inhibited about 50% after incubation of cells with the inhibitor for 30 min (Figure 2). Inhibitors of mitochondrial electron transport are very effective in inhibition of the ATP-dependent basal proton release, but have very little effect on the ferricyanide induced proton release (Figure 3).

In summary, the data of this study support the conclusion that H^+ excretion by carrot cells is generated by two separate mechanisms: 1) the action of the plasma membrane H^+-ATase, and 2) transplasma membrane electron transport, measured as ferricyanide reduction by these cells. The proportion from each component varies with growth

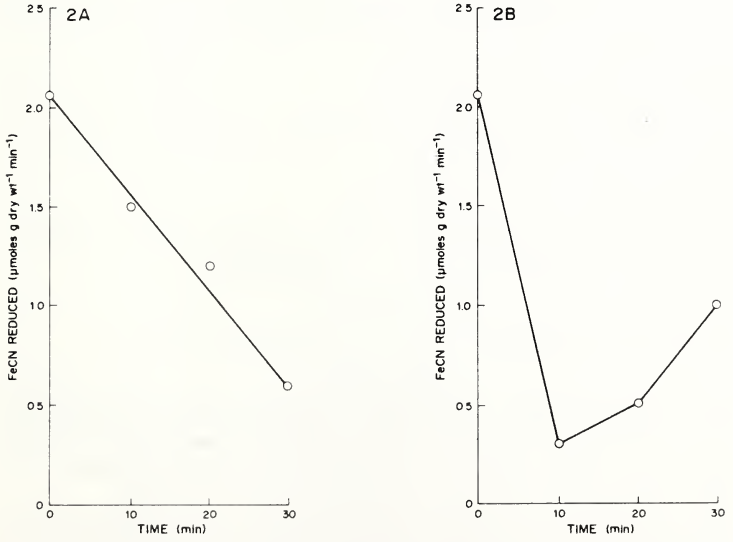

FIGURE 2A. The Effect of Incubating Cultured Carrot Cells with Diethyl Stilbestrol (100 μM) on Transmembrane Ferricyanide Reduction. The control rate was 2.06 μmoles FeCN reduced g dry wt.$^{-1}$ min^{-1}.

FIGURE 2B. The Effect of Incubating Cultured Carrot Cells with Dicyclocarbodiimide (100 μM) on Transmembrane Ferricyanide Reduction. The control rate was 2.06 μmoles FeCN reduced g dry wt.$^{-1}$ min^{-1}.

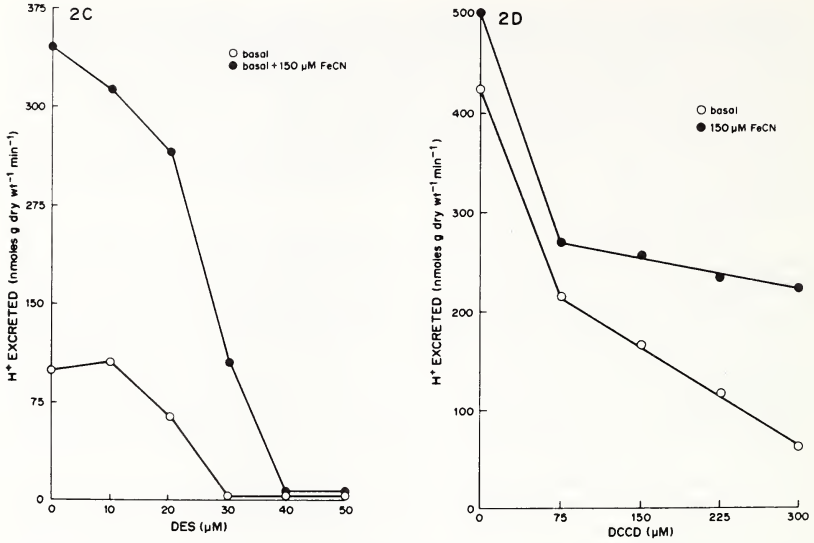

FIGURE 2C. The Effect of Diethyl Stilbestrol on H^+ Excretion by Cultured Carrot Cells. The basal rate of H^+ excretion was 101 nmoles H^+ excreted mg dry wt.$^{-1}$ min^{-1}; the rate with FeCN was 345.

FIGURE 2D. The Effect of Dicyclocarbodiimide on H^+ Excretion by Cultured Carrot Cells. The basal rate of H^+ excretion was 422 nmoles mg dry wt.$^{-1}$ min^{-1}; the rate with FeCN was 500.

conditions and the age of carrot cells. A study of factors which affect H^+ excretion during development is still in progress.

Abbreviations

DCCD, dicyclohexylcarbodiimide; DES, diethystilbestrol; HOQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; KCN, potassium cyanide.

Acknowledgments

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Literature Cited

1. Böttger, M. and H. Lüthen. 1986. Possible linkage between NADH oxidation and proton secretion in *Zea mays* L. roots. J. Exper. Bot. 178, 666-675.
2. Craig, T.A. and F.L. Crane. 1981. Evidence for a trans-plasma membrane electron transport system in plant cells. Indiana Acad. Sci. 90, 150-155.
3. Craig, T.A. and F.L. Crane. 1982. Hormonal control of a transplasma membrane electron transport system in plant cells. Indiana Acad. Sci. 91, 150-154.
4. Crane, F.L., I.L. Sun, M.G. Clark, C. Grebing and H. Löw. 1985. Transplasma-membrane redox systems in growth and development. Biochim. Biophys. Acta 811, 233-264.
5. Federico, R. and C.E. Giartosio. 1983. A trans-plasma membrane electron transport system in maize roots. Plant Physiol. 73, 182-184.
6. Ivankina, N.G. and V.A. Novak. 1980. H^+-transport across plasmalemma.

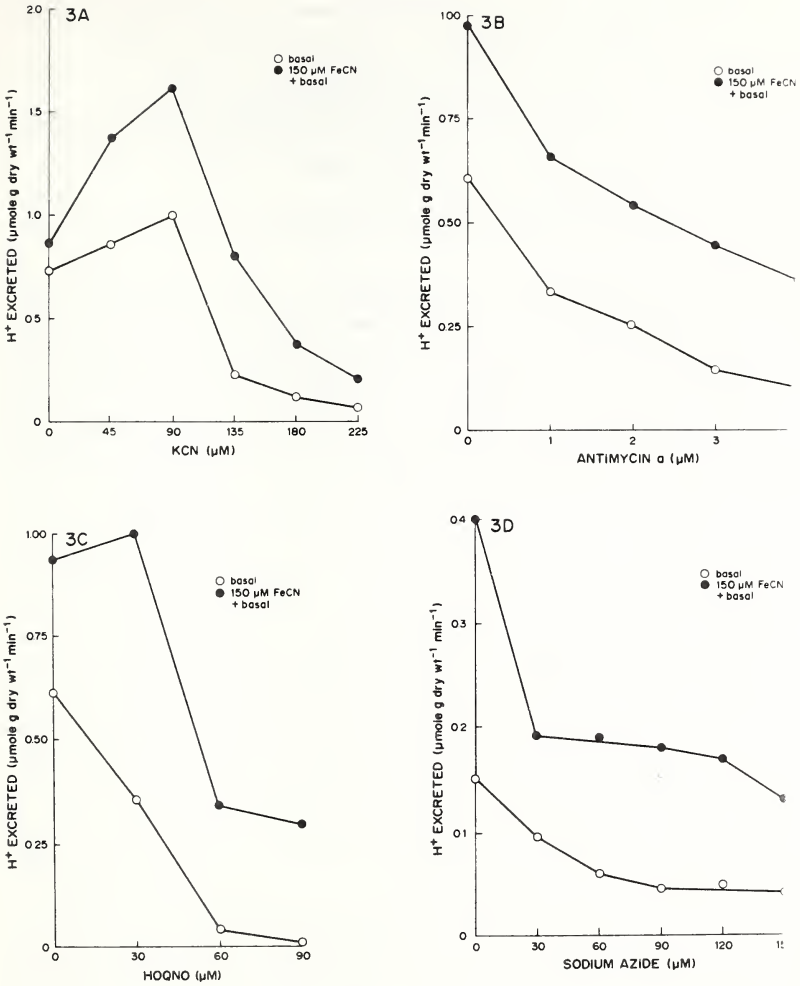

FIGURE 3. The Effect of Mitochondria Electron Transport Inhibitors on H^+ Excreti by Cultured Carrot Cells. A — KCN in concentrations indicated; B — antimycin a; — HOQNO; D — sodium azide. The assay conditions were as described in **Materi and Methods**. Note relative lack of inhibition of the ferricyanide-induced increment proton release, especially with antimycin a.

H^+-ATPase or redox-chain? In Plant Membrane Transport: Current Conceptu Issues (R.M. Spanswick, W.J. Lucas and J. Dainty, eds.), Elsevier, North-Holla Biomedical Press, Amsterdam, pp. 503-504.

7. Lin, W. and I.M. Møller. 1986. Membrane-bound NAD(P)H dehydrogenases higher plant cells. Ann. Rev. Plant Physiol. 37, 309-334.
8. Lüttge, U. and D.T. Clarkson. 1985. Mineral nutrition: plasmalemma and tonopl redox activities. Progress in Botany, vol. 47, Springer-Verlag, Berlin, pp. 73-

9. Marre, E. and A. Ballarin-Denti. 1985. The proton pumps of the plasmalemma and the tonoplast of higher plants. J. Bioenerg. Biomembr. 17, 1-21.
10. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.
11. Qui, Z.-S., B. Rubinstein and A.I. Stern. 1985. Evidence for electron transport across the plasma membrane of *Zea mays* root cells. Planta 165, 383-391.
12. Rubinstein, B., A.I. Stern and R.G. Stout. 1984. Redox activity at the surface of oat root cells. Plant Physiol. 76, 386-391.
13. Serrano, R. 1985. Membrane ATPase of Plants and Fungi. CRC Press, Boca Raton, Florida, pp. 80-129.
14. Sijmons, P.C., F.C. Lanfermeijer, A.H. de Boer, H.B.A. Prins and H.F. Bienfait. 1984. Depolarization of cell membrane potential during trans-plasma membrane electron transfer to extracellular electron acceptors in iron-deficient roots of *Phaseolus vulgaris* L. Plant Physiol. 76, 943-946.
15. Sze, H. 1985. H^+-translocating ATPases: advances using membrane vesicles. Ann. Rev. Plant Physiol. 36, 175-208.
16. Thom, M. and A. Maretzki. 1985. Evidence for a plasmalemma redox system in sugarcane. Plant Physiol. 77, 873-876.