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A Link Between Transport and Plasma Membrane Redox System(s) in Carrot Cells

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Abstract

Carrot (*Daucus carota* L.) cells grown in suspension culture oxidized exogeneous NADH. The NADH oxidation was able to stimulate K^+ ($^{86}Rb^+$) transport into cells, but it did not affect sucrose transport. *N,N'*-Dicyclohexylcarbodiimide, diethylstilbestrol, and oligomycin, which only partially inhibited NADH oxidation, almost completely collapsed the K^+ ($^{86}Rb^+$) transport. Vanadate, which is less effective as an ion transport inhibitor, was less effective in inhibiting the NADH-driven transport of K^+ ($^{86}Rb^+$). *p*-Fluormethoxycarbonylcyanide phenylhydrazone inhibits the K^+ transport over 90% including that induced by NADH. The results are interpreted as evidence that a plasma membrane redox system in root cells is closely associated with the ATPase which can drive K^+ transport. Because of the inhibitor effects, it appears that membrane components common to the redox system and ATPase function in the transport of K^+.

Key Words: Plasma membrane; NADH oxidation; potassium transport; ATPase inhibitors; proton gradient generation.

Introduction

The energy source for transport in plant cells is largely considered to be an electrochemical proton gradient (Hutchings, 1978a, b; Komor, 1977; Poole, 1978). The necessary proton gradient is generated on hydrolysis of intracellular ATP by plasmalemma ATPase(s), acting as proton pumps (Anderson *et al*, 1977; Giaquinta, 1977; Spanswick, 1982). The plasma membrane redox systems, present in various eukaryotic cells (Crane *et al.*, 1979) and shown in some cases to act as proton pumps (Craig and Crane, 1981; Crane *et al.*,

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1982a), may also be involved in energizing the transport mechanism (Crane *et al.*, 1982b; Löw and Crane, 1970). The presence of a transplasmamembrane electron transport and a concomitant pumping out of H^+ in carrot cells was reported earlier from our laboratory (Craig and Crane, 1982).

NADH-driven transport of amino acids in ascites tumor cells has been reported (Yamamoto and Kawasaki, 1981). An indication of ion transport mediated by an extra ATPase factor in renal tubules was given by Sachs (1977). Recently, NADH-driven potassium and phosphate transport in corn root protoplasts was reported (Lin, 1982, 1983). The oxidation of NADH added to the exterior of corn protoplasts is accompanied by increased K^+ transport. Therefore, it was thought that such a system should be able to drive the transport of metabolites and ions, if suitable substrate was added to carrot cells. Attempts were also made to delineate the membrane redox system involved in transport by incorporating ATPase inhibitors. DCCD,[4] DES, oligomycin, and vanadate are known to be effective inhibitors of plant ATPase(s) (Balke and Hodges, 1977; Cheeseman *et al.*, 1980; Leonard and Hodges, 1973; Sze and Churchill, 1981).

The results show that carrot cells are able to oxidize externally added substrates, e.g., NADH and NADPH, and such an oxidation is able to drive transport of potassium ions across the plasma membrane. It is concluded from our results that membrane components associated with plasma membrane ATPase act as a carrier of K^+ even when transport is energized by the external NADH oxidase.

Materials and Methods

Chemicals

^{86}RbCl and [U-^{14}C]sucrose were purchased from New England Nuclear. NADH, NADPH, glutathione (GSH), ascorbic acid, DCCD, DES, and oligomycin were from Sigma. All other chemicals were from Mallinkrodt or Fischer Scientific Company.

Cell Culture

A carrot cell culture line (*Daucus carrota* L.), obtained from Drs. P. M. Hasegawa and R. A. Bressan of the Purdue Horticulture Department, was grown in liquid suspension culture (Craig and Crane, 1981). Cells were harvested in the logarithmic phase of growth, washed twice with a solution

[4]Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; FCCP, *p*-fluoromethoxycarbonylcyanide phenylhydrazone; ATPase, adenosinetriphosphatase.

containing 0.2 M mannitol and 10 mM each of NaCl, KCl, and $CaCl_2$. Finally, the cells were suspended in the same solution and the suspension was continuously aerated. The aliquots were drawn at least 60 min after the cells had been transferred from growth media to decrease effects of residual hormone from the growth media. For measurement of dry weight a separate aliquot of cells was washed twice with distilled water, dried under an air jet, and the moisture was removed by heating in a 90° oven to constant weight.

Measurement of NADH Oxidation

The rate of NADH oxidation by cells was measured by recording the decrease in absorption at 340 nm using an Aminco DW-2a spectrophotometer in the dual-beam mode with a reference at 500 nm at 25°C (Cherry *et al.*, 1981). The cell suspension was incubated at pH 7.0 in 13.0 mM Tris-Mes buffer at least 5 min prior to the addition of NADH (0.25 mM) and stirred continuously with a magnetic stirring assembly. A millimolar extinction coefficient of 6.23 was used for NADH.

Measurement of Transport

Radioactive-labeled substances were used for monitoring transport. 4 × 10.0 ml cell suspension (packed cell volume 2.0–2.5 ml per 10 ml) was removed from growth flasks and transferred to four graduated tubes. The suspension was centrifuged for 2.0 min in a clinical centrifuge and the supernatant removed by suction. The packed cells were suspended in 0.2 M mannitol containing 10 mM each NaCl, KCl, and $CaCl_2$ to make 10 ml total volume. After another wash the cells were combined in 40 ml of mannitol–salt media in an Erlenmyer flask and shaken for 2 to 3 hours for aeration and to decrease endogenous metabolism. Potassium was omitted from the mannitol–salt solution when cells were to be used for K^+ ($^{86}RbCl$) transport. After centrifugation the cells were suspended in the same medium buffered with 13 mM Tris-Mes, pH 7.0. The reaction was initiated by the addition of labeled substrate at 25°C. Aliquots (1 ml) were removed at different intervals, filtered quickly under suction through a 25-mm Metricell membrane filter type GN-4, pore size 0.8 μm, and washed by ice-cold suspension medium containing the same concentration of "cold" substrate as in the reaction mixtures. The cell mass together with the membrane filter was transferred to a known volume of water, boiled for 5 min in a water bath, and centrifuged. The radioactivity was then measured in the supernatant. Sucrose transport was measured by using 20 mM [U-^{14}C]sucrose. Specific radioactivity was 5 mCi per mole. For measuring potassium transport, cells were washed and aerated in a potassium-free medium. $^{86}RbCl$ was used as the label for monitoring potassium transport. The concentration of potassium in the reaction system

Table I. Effect of ATPase Inhibitors on the Rate of NADH Oxidation by Carrot Cells[a]

Assay conditions	Inhibitor concentration	Activity (nmol/min/mg dry wt.)	Change from control (%)
Control	—	2.33 ± 0.11	—
+ Oligomycin	1.0 μg/ml	1.87 ± 0.01	−19.7
+ DES	10^{-4} M	2.04 ± 0.09	−12.5
+ DCCD	10^{-4} M	1.12 ± 0.02	−51.9
+ Vanadate	2×10^{-4} M	2.04 ± 0.03	−12.5

[a]Cells were treated with inhibitors 5–7 min prior to the addition of NADH (0.25 mM). Control runs were treated identically with equivalent amounts of solvents. Other details are described under Materials and Methods. Activity was measured by a decrease in absorbance at 340 nm. The values are average ±SE of four identical runs.

was 50 μM, and specific radioactivity was nearly 5 Ci per mole. Counter efficiency was 93 and 98% for ^{14}C and ^{86}Rb, respectively.

Parallel control runs were always done, since there is variation in basal values with different batches of cells.

Results

The rate of NADH oxidation by carrot cells and the effect of ATPase inhibitors on this activity is reported in Table I. NADH oxidation, in general, is inhibited to various degrees, and maximum inhibition is given by DCCD. To test whether NADH oxidation as observed in Table I was not just its uptake into the cells, the rate of O_2 uptake by cells was measured under similar conditions, and the results are compared in Table II.

NADH stimulates O_2 uptake, and this is inhibited by ATPase inhibitors. The pattern of inhibition of stimulated O_2 uptake is nearly the same, as is seen

Table II. Effect of ATPase Inhibitors on the Rate of NADH-Stimulated Oxygen Consumption by Carrot Cells[a]

Assay conditions	Inhibitor concentration	Rate (nmol O_2/min/mg dry wt.)	Change from control (%)
Control	—	4.56 ± 0.08	—
+ NADH	—	5.84 ± 0.15	+28.1
+ NADH + oligomycin	1.0 μg/ml	5.52 ± 0.10	+21.2 (−25.0)
+ NADH + DES	10^{-4} M	5.67 ± 0.09	+24.3 (−13.3)
+ NADH + DCCD	10^{-4} M	5.08 ± 0.09	+11.4 (−59.3)
+ NADH + vanadate	2×10^{-4} M	5.63 ± 0.08	+23.5 (−16.4)

[a]The oxygen uptake was measured by a Clark type electrode (YSI) at 25°C in 13.0 mM Tris-Mes buffer at pH 7.0. The details of treatment of cells with inhibitors were the same as described in Table I. NADH concentration was 0.25 mM. The values are average ±SE of four identical runs. The values in parentheses represent the percent inhibition of NADH-stimulated oxygen consumption.

in Table I. These observations suggest that NADH oxidation and its inhibition are at the plasma membrane level. In addition, about 56% of the NADH-stimulated rate of O_2 uptake is retained in the presence of cyanide. Quinacrine (10^{-3} M) inhibits 43% NADH-stimulated O_2 uptake (Lin, 1982). Antimycin A (10^{-6} M) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (10^{-5} M) did not inhibit NADH cytochrome *c* reductase activity observed with cells. The cytochrome oxidase activity (nmol cyt *c* oxidized/min/mg dry weight of cells) was 9.0, and there was practically no detectable succinate cytochrome *c* reductase activity. These observations rule out any possible mitochondrial contamination in cell suspensions. However, it is to be noted from Tables I and II that even in the presence of ATPase inhibitors, significant oxidation of NADH continues. The oxidation reaction does not seem to generate superoxide, because superoxide dismutase (7.68 units/ml) did not inhibit the reduction of external cytochrome *c*.

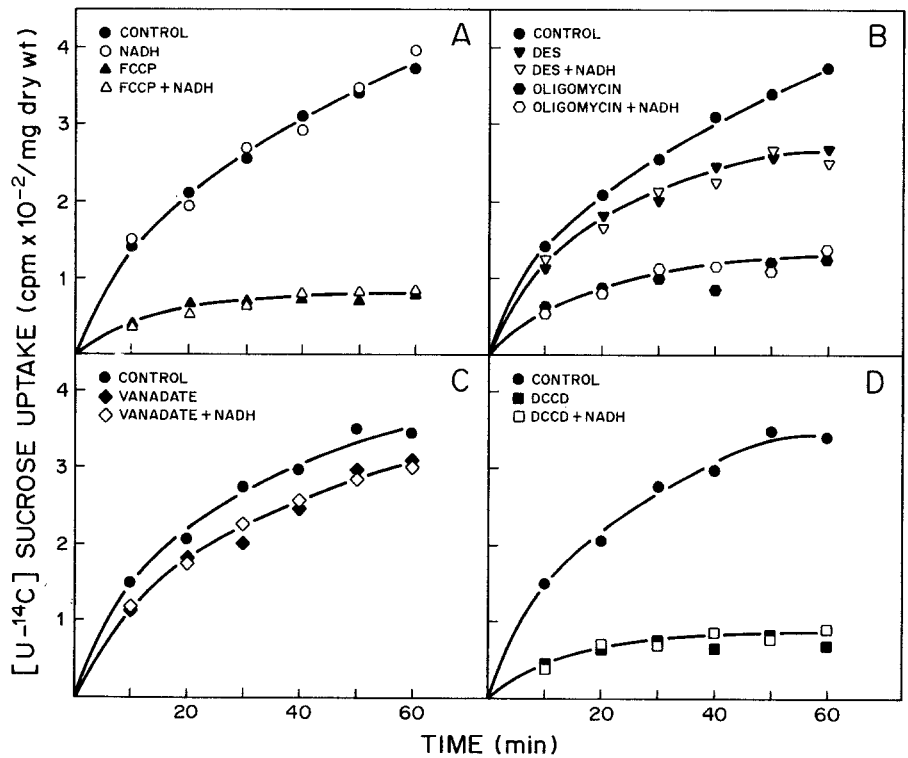

Fig. 1. Time course of sucrose transport in carrot cells. DCCD (10^{-4} M), DES (10^{-4} M), vanadate (2×10^{-4} M), and oligomycin (1 μg/ml) inhibited the uptake (B, C, and D). NADH (0.25 mM) had no effect, and FCCP (10^{-5} M) collapsed the transport (A). Details of the procedure are described under Materials and Methods.

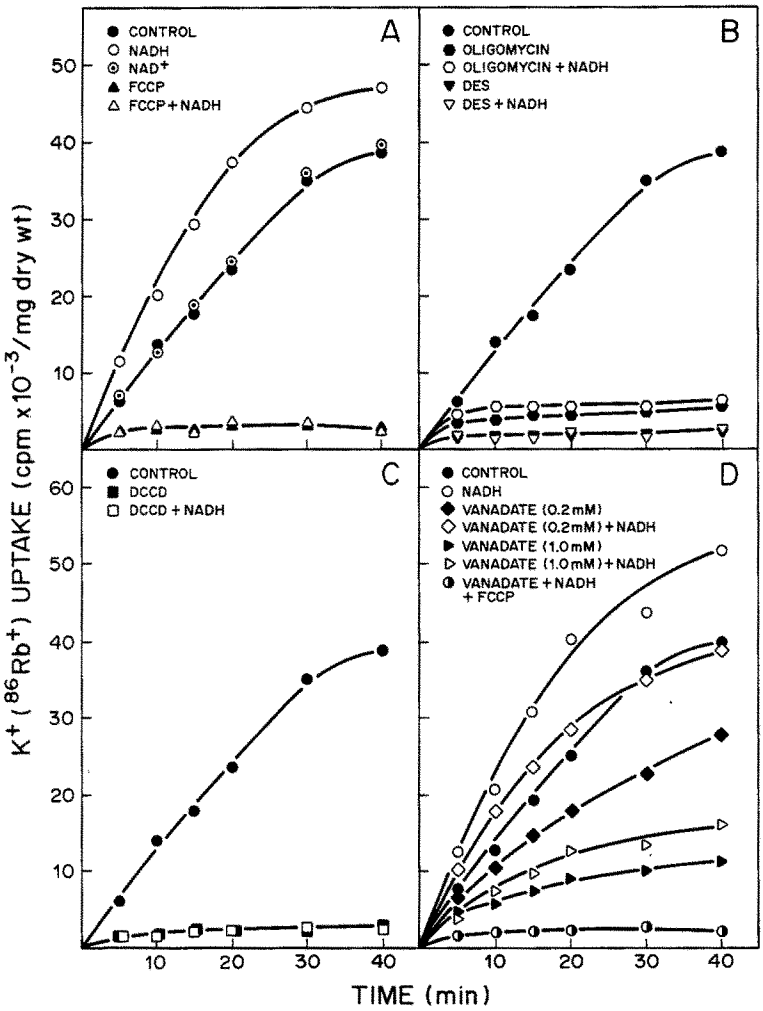

Fig. 2. Time course of NADH-stimulated K^+ ($^{86}Rb^+$) uptake under normal and inhibitory conditions by carrot cells. The concentrations of NADH and inhibitors were the same as in Fig. 1, and details of the procedure are described under Materials and Methods. Concentration of NAD^+ was 0.25 mM.

It was thought interesting to study the transport of sucrose and potassium in NADH-oxidizing cells because they are good models for testing nonionic and ionic transport under culture conditions. The results of these studies, in the presence and absence of NADH and with ATPase inhibitors, are presented in Figs. 1 and 2.

It is seen from Fig. 1 that NADH oxidation does not affect sucrose transport under normal or inhibitory conditions. Added NADH enhances K^+

transport (Fig. 2A). The incorporation of FCCP collapses this transport as in control cells, thus indicating it to be tightly linked to metabolic energy. Since NAD^+ does not affect K^+ transport, the enhanced transport is linked to NADH oxidation.

In measuring K^+ transport the concentration of potassium in the external medium was kept low, because under these conditions K^+ transport is quite sensitive to ATPase inhibitors and the effects of diffusion potential as seen at higher concentrations are not involved (Cheeseman *et al.*, 1980). Various ATPase inhibitors, with the exception of vanadate, showed strong inhibition of the transport process, and the addition of NADH to such a system did not enhance the transport rate. In the case of vanadate, where two widely varied concentrations were tested (Fig. 2D), the inhibition of K^+ transport was not complete. The addition of NADH to this system enhanced the transport, and it was collapsed by FCCP. This suggests that vanadate, unlike other inhibitors, probably does not completely block a component of the K^+ transporting system and allows a partial response to NADH. These results indicate that the plasma membrane redox system in carrot cells is involved in driving ion transport.

Besides NADH oxidase, various redox enzymes are known to be present in plasma membrane systems (Crane *et al.*, 1980; Goldenberg, 1980; Mukherjee and Lynn, 1977; Ormstad *et al.*, 1979). It was therefore desirable to compare the rate of NADH oxidation with some other substrates. Table III compares the effect of various substrates, measured as stimulated O_2 uptake by carrot cells. It is seen that NADH gives the highest oxidation rate, followed by glutathione (GSH), NADPH, and ascorbate. The enhanced transport of K^+ driven by NADH was also compared to transport during oxidation of these alternative substrates, and the values are reported in Table IV. Only NADH and NADPH oxidation stimulates K^+ uptake, while glutathione had no effect and ascorbate inhibited the K^+ transport. It can be seen that the highest rate of NADH oxidation is parallel to the maximum stimulation of K^+ uptake.

Table III. Comparative Rates of Oxygen Uptake by Carrot Cells in the Presence of Various Substrates[a]

Assay condition	Rate (nmol/min/mg dry wt.)	Change from control (%)
Control	5.47 ± 0.11	
+ NADH	7.20 ± 0.17	+31.6
+ NADPH	5.98 ± 0.10	+9.3
+ Ascorbic acid	5.84 ± 0.11	+6.8
+ Glutathione (GSH)	6.04 ± 0.11	+10.4

[a]The method and conditions of assay were the same as described in Table II, except a separate batch of cells was used. The concentration of each substrate was 0.25 mM. The values are average ±SE of four identical runs.

Table IV. Effect of NADH, NADPH, Glutathione (GSH), and Ascorbic Acid on the Uptake of K^+ ($^{86}Rb^+$) by Carrot Cells[a]

Assay condition	Uptake [nmol K^+ ($^{86}Rb^+$)/mg dry wt.]	
	10 min	15 min
Control	0.78	1.81
+ NADH	1.06 (+35.9)	2.45 (+35.4)
+ NADPH	0.93 (+19.2)	2.26 (+24.9)
+ Glutathione (GSH)	0.79 (+1.3)	1.48 (−18.2)
+ Ascorbic acid	0.40 (−48.7)	0.52 (−71.3)

[a]The concentrations of reduced substrates were as given in Table III. The details of transport measurements are described under Materials and Methods. The values in parentheses are percent change from control.

Lack of response with glutathione suggests that it was, probably, oxidized inside the cells. How ascorbate caused inhibition is, presently, not known. These results indicate that NADH/NADPH oxidation stimulates K^+ transport, when added to an external site which is not seen with the other substrates tested. According to Lin (1982) the rate of NADPH oxidation by corn root protoplasts was 10% of the rate of NADH oxidation, and whether this oxidation was linked to ion transport is not reported. With carrot cells, the rate of NADPH oxidation can be 30% of the rate of NADH oxidation, and NADPH oxidation is accompanied by K^+ transport.

Discussion

The present observations showing NADH oxidation by carrot cells and linkage of this oxidation with K^+ transport are in accord with the earlier reports on corn root protoplasts and ascites tumor cells (Lin, 1982; Yamamoto and Kawasaki, 1981), where oxidation of externally applied NADH is linked to the transport of mineral ions and amino acids, respectively. The present work with sucrose and potassium transport is, therefore, a step forward in establishing that a membrane redox system has a specific role in driving ion transport. It is now generally believed that in plant roots an active H^+ extrusion, powered by a plasmalemma-bound ATPase, is operative and provides a proton gradient for K^+ transport. Whether K^+ is carried by the H^+ pump itself or by a separate carrier is still unsettled (Läuchli, 1979; Poole, 1978; Ratner and Jacoby, 1976). However, there are reports that a plasmalemma-bound ATPase may act as a carrier of K^+ (Bowling, 1976; Hodges, 1976; Läuchli, 1979; Leonard and Hotchkiss, 1976). The transport of K^+ driven by NADH suggests that NADH oxidation is capable of at least part of the role of membrane-bound ATPase in providing the necessary H^+ gradient required for K^+ transport. Lin (1982) has shown that FCCP, which destroys

proton gradients, inhibits endogenous and NADH-dependent K^+ uptake. ATPase inhibitors, with the exception of vanadate, inhibit K^+ transport almost completely. This indicates that components of the plasmalemma ATPase in carrot cells is required in K^+ uptake. It is to be noted (Tables I and II) that, in general, there is a significant NADH oxidation even in the presence of ATPase inhibitors, but the oxidation alone fails to drive K^+ transport (Fig. 2B and C). Vanadate is an exception, because the addition of NADH in its presence always shows enhanced K^+ transport (Fig. 2D). This may be due to an incomplete ATPase inhibition by vanadate. These results, therefore, lead to the conclusion that, like other transport systems, the K^+ transport in carrot cells involves two components: the first is required for the supply of energy in the form of a proton gradient, and the second acts as a carrier for ion movement. The proton gradient is generated either by the plasmalemma ATPase by hydrolyzing cellular ATP and/or under certain conditions, as shown here, by membrane redox systems. It appears from these experiments that the role of the K^+ carrier, which has to be very specific, can be played by a protein associated with the membrane-bound ATPase. The exact mode of energization of K^+ transport by NADH oxidation is yet to be elucidated, but, in agreement with earlier views (Läuchli, 1979), a transmembrane proton gradient may be the basis for the K^+ transport. This gradient may be developed either by action of an ATPase or through the NADH oxidase on the membrane (Lin, 1982, 1983).

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