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Electron donation to the plasma membrane redox system of cultured carrot cells stimulates proton release

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Membrane-permeable electron donors, duroquinol, diphenylcarbazide, pyrocatechol and *tert*-octylcatechol, promoted both reduction of an impermeant electron acceptor and proton transport with cultured carrot cells. These cells were preloaded with electron donors for 15, 30, 45 and 60 min. Aliquots of cells were removed at various times, washed free of excess electron donors and assayed for their effect on transplasma membrane redox with impermeable hexacyanoferrate (HCF III) as the electron acceptor and for simultaneous H^+ excretion in the presence of hexacyanoferrate. All four electron donors stimulated HCF III reduction and associated H^+ excretion. Below a rate of hexacyanoferrate reduction of 6 μmol/g dry wt. per min, the ratios of H^+/e^- were between 0.3 and 1 with low concentrations (0.1 mM) of the added electron donors. When hexacyanoferrate reduction exceeded 6 μmol/g dry wt. per min, proton release began to cascade to give ratios of 1 to 3, suggesting activation of an H^+-ATPase or a proton transporter. This behavior by cultured carrot cells indicates that a certain threshold of proton concentration in a limited membrane domain must be reached in order for the proton channel to be opened.

Introduction

NAD(P)H is the natural electron donor for the plant plasma membrane electron transport chain [1–3]. It is oxidized presumably on the cytoplasmic surface of the membrane and the electrons cross to the outside of the cell via undetermined electron carriers. The electrons may [1,4–10] or may not [11–15] be accompanied by proton excretion. Some authors have suggested that the protons produced by NADH oxidation activate the plasma membrane H^+-ATPase, to account for stimulated proton excretion [11,16]. Since NADH is impermeable to plant plasma membranes, extracellular NADH cannot be used as a transmembrane redox electron donor to whole cells. However, intracellular NADH is thought to donate electrons to an outside enzyme, the hormone-sensitive NADH oxidase recently isolated from soybean hypocotyls [17]. Therefore, in this study using whole carrot cells, other, more permeable exogenously added electron donors were investigated in an effort to experimentally increase electron donation from the inside. It was found that duroquinol, diphenylcarbazide, pyrocatechol and *tert*-octylcatechol stimulated transmembrane redox reactions and associated H^+ excretion with HCF III as an impermeable electron acceptor on the outside of cells. Higher concentrations of electron donors (5–10 mM) induced a proton cascade with ratios H^+/e^- of greater than 1. This introduces a new concept in regard to proton excretion by plant cells, namely, the forced opening of proton channels.

Methods

Carrot cells were grown in tissue culture on Murashige and Skoog's medium without agar as previously described [18]. After 4 to 8 days, while still in the log phase of growth, cells were harvested by centrifugation in an International unrefrigerated table model centrifuge at $200 \times g$ for 2 min. They were washed 3 times with sucrose-salts solution (0.1 M sucrose with 10 mM KCl, 10 mM NaCl and 10 mM $CaCl_2$) and finally suspended in 50 ml of this solution. Cells were kept aerated on a reciprocal shaker until ready for use.

Abbreviations: DPC, diphenylcarbazide, DQ, duroquinol; HCF III, hexacyanoferrate or potassium ferricyanide; TOC, *tert*-octylcetachol.

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For incubation with electron donors, 5 ml aliquots of cells in 15-ml centrifuge tubes, supplied with the appropriate electron donor of a chosen concentration were incubated on a reciprocal shaker for 15, 30, 45 and 60 min. When time was up, the centrifuge tubes containing cells with electron donors were removed from the shaker, diluted with 10 ml of fresh sucrose-salts solution and centrifuged for 2 min as before. After removal of the supernatant, the cells were again suspended in 5 ml sucrose-salts solution, and 0.1 ml portions were assayed simultaneously for HCF III reduction and H^+ excretion.

HCF III reduction by carrot cells was measured with an Aminco DW-2A spectrophotometer in the dual mode as the difference in absorbance at 420 nm minus 500 nm (reference) at 24°C [18]. Reaction rates were recorded with a Linear recorder. The reaction mixture consisted of carrot cells (about 1 mg dry wt.), a mixture of 25 mM Tris-Mes (pH 7) and sucrose-salts solution to bring the volume up to 1.5 ml. After a 3 min preincubation period, the reaction was started by adding 100 μM HCF III. Reaction rates were calculated using a millimolar extinction coefficient of 1 for HCF III ($E_{420} = 1$ $mM^{-1} \cdot cm^{-1}$).

H^+ excretion by carrot cells was measured with a Corning combination pH electrode and a Corning pH meter as previously described [8]. The water-jacketed reaction chamber was kept at 24°C, and reactions were under subdued fluorescent room lighting. A stream of air was bubbled through the reaction chamber, while the reaction mixture was stirred with a small bar on a magnetic stirrer. A reaction mixture consisted of 4.9 ml sucrose-salts solution, 0.1 ml cells (approx. 1 mg dry wt.), 25 μM potassium phosphate buffer (pH 7) and 25 μl ethanol. The reaction was recorded with a linear recorder for 5 min to obtain the basal rate, or H^+

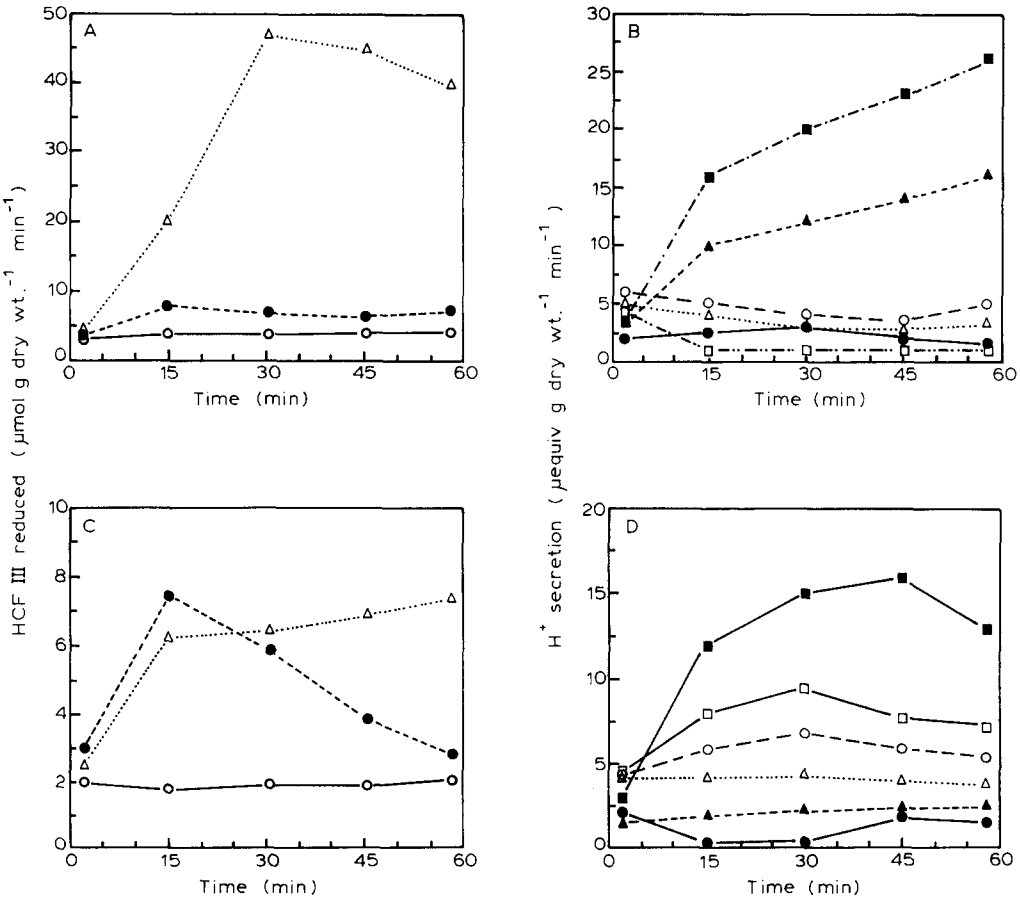

Fig. 1. The effect of pre-incubating carrot cells with different concentrations of duroquinol (DQ) and diphenylcarbazide (DPC) on transmembrane HCF III reduction and associated H^+ excretion. Excess DQ and DPC was removed from cells by washing with sucrose-salts solution before assays. HCF III reduction and H^+ excretion assays were performed as described in Methods. (A) HCF III reduction by cells preloaded with DQ; (B) H^+ excretion by cells preloaded with DQ; (C) HCF III reduction by cells preloaded with DPC; (D) H^+ excretion by cells preloaded with DPC. Symbols: (A) cells preloaded with 0.1 mM DQ (○), 1 mM DQ (●) and 5 mM DQ (△); (B) cells preloaded as in 9A) but open symbols indicate basal rate of H^+ excretion with 0.1 (○), 1 (△) and 5 (□) mM DQ, while closed symbols H^+ excretion in presence of HCF III with comparable concentrations of DQ; (C) cells preloaded with 0.1 mM DPC (○), 1 mM DPC (●) and 10 mM DPC (△); (D) cells preloaded as in (C), but open symbols indicate basal rate of H^+ excretion with 0.1 mM DPC (○), 1 mM DPC (△) and 10 mM DPC (□), while closed symbols H^+ excretion in presence of HCF III with comparable concentrations of DPC.

excretion presumed due to the action of the plasma membrane H^+-ATPase. After 5 min, 100 μM HCF III was added to measure the contribution of plasma membrane redox to H^+ excretion. After 5 more min, a known amount of HCl from a 0.01 M stock solution was added to calibrate the assay.

Leakage from cells preloaded with electron donors was determined for treated cells, washed free of exogenous electron donors. They were allowed to equilibrate for 10 min in sucrose-salts solution on a reciprocal shaker (30 pendulum motions per min). Then the preloaded cells were removed by centrifugation at 200 × g for 2 min and the supernatants assayed for HCF III reduction and H^+ excretion as described above.

Results

Results from the four different electron donors added to cultured carrot cells were similar (Fig. 1). When cells were preloaded with the electron donors in various concentrations and the excess was washed off from the outside of cells, DQ for example, at 0.1 and 1 mM had little effect on transmembrane HCF III reduction by carrot cells, whereas 5 mM DQ stimulated both HCF III reduction and HCF III-associated H^+ excretion almost 10-fold after 15 min (Fig. 1A, B). With longer incubations, however, proton excretion was accelerated disproportionately.

DPC also donated electrons to plasma membranes of carrot cells (Fig. 1C), giving about 3-fold stimulation over control rates. Of the four electron donors used, DPC was the only electron donor which also stimulated the basal rate of H^+ excretion (Fig. 1D). With the other three electron donors, the basal rate of H^+ excretion was inhibited.

Pyrocatechol(*o*-dihydroxybenzene) stimulated HCF III reduction by carrot cells in concentrations of 1–10 mM (Fig. 2A). The redox-associated H^+ excretion was

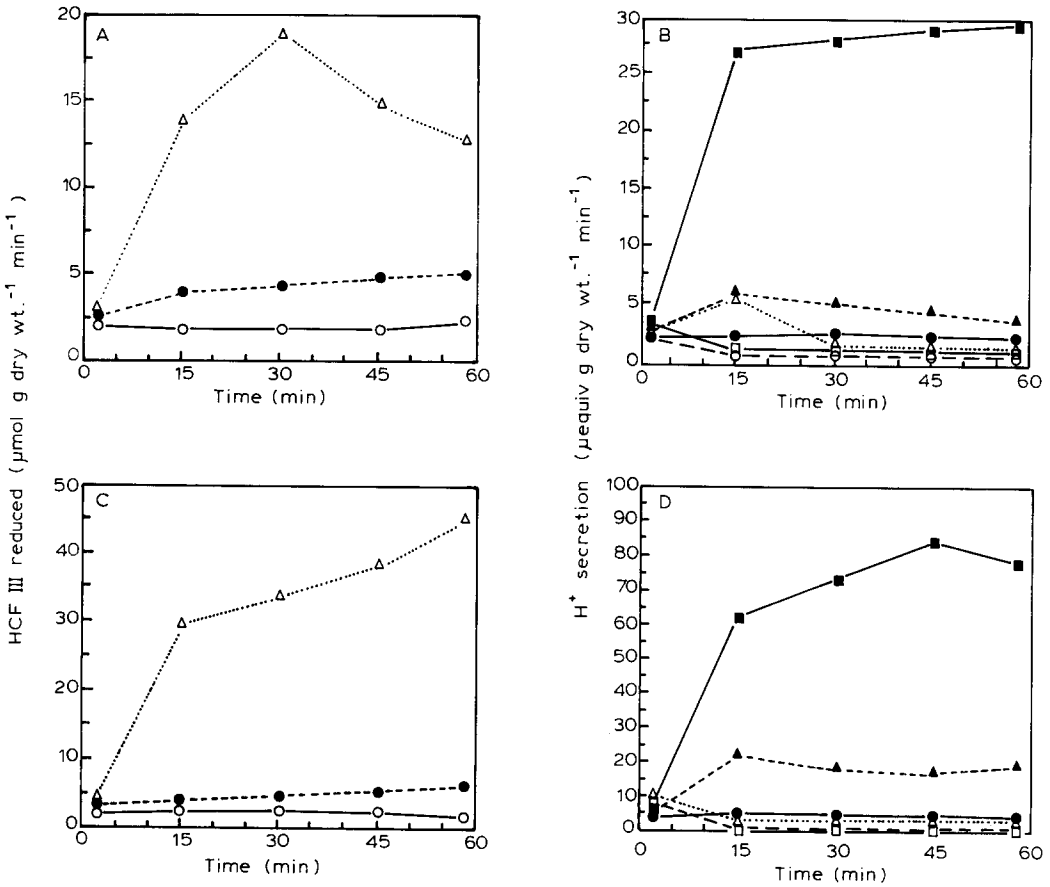

Fig. 2. The effect of incubating carrot cells with various concentrations of pyrocatechol and TOC on transmembrane HCF III reduction and associated H^+ excretion. Excess catechols were removed from cells by washing with sucrose-salts solution before assays. HCF III and H^+ excretion assays were performed as described in Materials and Methods. (A) (HCF III reduction by cells preloaded with pyrocatechol; (B) H^+ excretion by cells preloaded with pyrocatechol; (C) HCF III reduction by cells preloaded with TOC (D) H^+ excretion by cells preloaded with TOC. Symbols: (A) cells preloaded with 0.1 mM pyrocatechol (○), 1 mM PC (●), 10 mM PC (△); (B) cells preloaded as in (A) but open symbols indicate basal rate of H^+ excretion with 0.1 mM PC (○), 1 mM PC (△), 10 mM PC (□), while closed symbols H^+ excretion in presence of HCF III with comparable concentrations of DQ; (C) cells preloaded with 0.1 mM TOC (○), 1 mM TOC (●), and 10 mM TOC (△); (D) cells preloaded as in (C), but open symbols indicate basal rate of H^+ excretion with 0.1 mM TOC (○), 1 mM TOC (△) and 10 mM TOC (□), while closed symbols H^+ excretion in presence of HCF III with comparable concentrations of DPC.

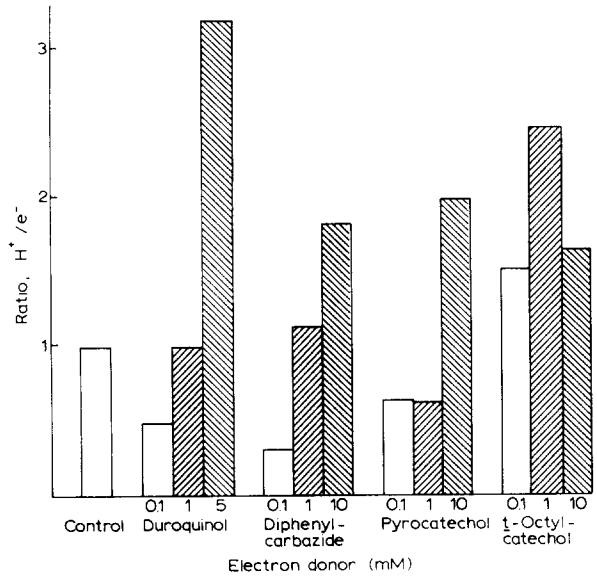

Fig. 3. Ratios of proton excretion to HCF III reduction in carrot cells preloaded with different electron donors at different concentrations. The carrot cells were incubated for 60 min, then washed and assayed for H^+-efflux and HCF III reduction.

stimulated, but the basal rate of H^+ excretion was inhibited (Fig. 2B). TOC, a lipophilic catechol with an eight-carbon side-chain, was an excellent electron donor for HCF III reduction by carrot cells (Fig. 2C). Reduction rates were increased from 3.2 μmol HCF III reduced/g dry wt. per min to nearly 50. HFC III-associated H^+ excretion increased about 20-fold with 10 mM TOC as the electron donor. Yet, the basal rate of H^+ excretion was completely inhibited (Fig. 2D).

The ratio between H^+ excreted and electrons transferred through the electron transport chain of the plasma membrane of carrot cells was near unity for control cells or less than 1 for cells treated with low concentrations of some of the electron donors (Fig. 3). However, at rates of hexacyanoferrate reduction greater than 6 μmol/g dry wt. per min, proton release exceeded electron transfer with a ratio of H^+/e^- of more than 3 for DQ and approx. 2 for the other electron donors tested (Fig. 3).

Discussion

Besides serving as an internal electron donor for transmembrane redox reactions, NADH may also donate electrons to activate an outside NADH oxidase(s) [16,19–25]. A hormone-sensitive NADH oxidase [17] and an outside NADH oxidase have been isolated [20,21,26].

Membranes are not permeable to NADH, so NADH was not used as an electron donor to whole carrot cells in this study. However, DQ, which stimulates NADH oxidase of *Cucurbita* microsomes [27,28], is permeable to cells and was used as a donor for maize root plasma membranes [29,30]. The present measurements of DQ-stimulated activity differed from those carried out previously in that cells were preloaded with DQ for 15, 30, 45 and 60 min. Excess DQ was removed from the outside of cells by washing with sucrose-salts before assays. Under such conditions, higher concentrations of DQ (5 mM) were necessary to donate electrons to HCF III reductase and associated H^+ excretion (Fig. 1A, B) than required by isolated membrane preparations. Pyrocatechol and a more lipophilic derivative of catechol, TOC, also were effective electron donors for HCF III reductase that stimulated H^+ excretion (Figs. 2A–2D). With DQ, pyrocatechol and TOC as electron donors, preloading cells inhibited the basal proton excretion. An exception was DPC, which stimulated basal H^+ excretion, as well as that induced by HCF III (Figs. 1C,D).

Since DPC donates electrons in spinach chloroplasts between the water oxidation site and Photosystem II reaction center [31], as does catechol, it is assumed that the redox potentials of DPC and pyrocatechol are positive and lie in the range of around + 400 mV, whereas the redox potential of duroquinol is +57 mV [32]. Another explanation may reside in the difference between DPC and the quinol-type electron donors which release H^+ upon oxidation easily, whereas DPC does so only very slowly. If released protons are concentrated in special membrane domains, the threshold for proton channel opening is reached faster with the quinone-type electron donors.

A comparison of the ratios between plasma membrane redox with HCF III as the electron acceptor and H^+ excreted in presence of HCF III should yield a ratio of 1, which is typically seen in studies with *Elodea* leaves [33] and cultured asparagus mesophyll cells [7,9,10]. However, in maize and bean roots, ratios of about 0.5 have been obtained [34]. With cultured carrot cells, average ratios between H^+ excreted and HCF III reduced were 1 (Fig. 3) in the control preparation not incubated with exogenous electron donors, but less than 1 with low concentrations of some of the electron donors. After cells were incubated for up to 60 min with DQ, DPC, pyrocatechol or TOC, and washed free of these donors before assays for HCF III reduction and proton excretion, the ratios of H^+/e^- changed from about 2 to more than 3 (Fig. 3). Since a higher ratio of protons to electrons implies a higher rate of H^+ excretion than HCF III reduction by these cells, what is the origin of the extra protons? Activation of the $[H^+]$-ATPase is unlikely, since the basal rate of H^+ excretion is only slightly stimulated by DPC (Fig. 1D) or totally inhibited by DQ, pyrocatechol or TOC. One possibility would be the opening of a proton channel from some intramembrane domain or even from the cytoplasm to the outside of cells. This might be expected to alter both the pH of the cell sap and the membrane potential [14,35], but these two parameters have not yet been

investigated in this context. A leaching of proton donors to the assay medium and a subsequent oxidation by HCF III would result in a 1 : 1 ratio. The substantially higher ratio indicates that the oxidation occurs in the cytoplasm and that this event is a transmembrane electron and proton transfer. Leaking from preloaded cells was minimal, 10% by catechol and 0 by the other electron donors. One possibility consistent with the observations is that protons from the oxidation of the donors themselves, i.e., quinol → quinone + $2H^+$, lowers cytoplasmic pH and thus activates a H^+ release mechanism, possibly by an allosteric mechanism. In any event, it appears that extra protons are generated regardless of the pumping mechanism.

Acknowledgements

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