Communication

Generation of a Membrane Potential by Electron Transport in Plasmalemma-Enriched Vesicles of Cotton and Radish

MIRIAM HASSIDIM, BERNARD RUBINSTEIN*, HENRI R. LERNER, AND LEONORA REINHOLD
Botany Department, The Hebrew University of Jerusalem, Jerusalem 91904, Israel (M.H., H.R.L., L.R.); and Botany Department and Program of Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003 (B.R.)

ABSTRACT

Plasmalemma-enriched vesicles were isolated from cotton roots (Gossypium hirsutum L. cv Acala San Jose 2) and from germinating radish seeds (Raphanus sativa L. cv Tondo Rosso Quarantino). When 100 mmol/L ascorbate was added to the grinding medium, the addition of ferricyanide to either preparation led to an inside positive membrane potential as measured by the accumulation of thiocyanate. It is suggested that electrons from ascorbate were being transported electrogenically across the membrane to ferricyanide, resulting in an accumulation of protons within the vesicle. The redox activity of the vesicles has some similarities to that occurring in intact cells, thus providing a simpler system to study the components and effects of transmembrane electron transport.

Electron transport can be detected at the plasmalemma of a variety of plant and animal cells, and may occur either in the plane of the membrane, when both a nonpermeating donor and acceptor are added, or across the membrane when a cytosolic donor is oxidized by an extracellular acceptor (8). This redox activity has been associated with processes at the cell surface, such as certain light-mediated events (10, 13, 15, 19). Fe uptake (4), H+ extrusion (5, 22), and fluxes of other solutes (12, 13, 16, 22).

However, little is known about the redox components, the identity of the natural acceptor, or the linkage between electron transport and processes located at the plasmalemma, so investigations have been carried out on plasmalemma-enriched preparations. Studies so far have determined light-induced absorbance changes (14, 25), as well as NAD(P)H oxidase and NAD(P)H oxidoreductase activities (2, 3, 7, 17, 23). The latter activities may be characteristic of electron transport in the plane of the membrane, since both the donor and acceptor are located at the same membrane surface.

In the report which follows, we describe vesicular preparations from two very different plant sources, enriched in plasmalemma and depleted in mitochondria and other membranes. The vesicles not only have oxidoreductase activity, but transport of electrons across the vesicle membrane is inferred. This transmembrane redox activity is compared to that observed in intact systems.

MATERIALS AND METHODS

Preparation of Vesicles. Plasmalemma-enriched vesicles were isolated from roots of 3-week-old hydroponically grown cotton seedlings (Gossypium hirsutum L. cv Acala San Jose 2) by differential centrifugation and by separation on a sucrose step gradient according to the methods of Hassidim et al. (11). Microsomes from radish seeds (Raphanus sativa L. cv Tondo Rosso Quarantino), which had been imbied in distilled H2O with shaking for 24 h, were prepared by the methods of Rasi-Caldogno et al. (21), except that 100 mm ascorbic acid was substituted for β-mercaptoethanol in the grinding medium and ATP was omitted. Criteria used by Rasi-Caldogno et al. (21) as evidence for plasmalemma enrichment (sensitivity of the ATP-induced H+ pumping to vandate, insensitivity to nitrate, nucleotide specificity, etc.) were found to hold true for our preparation as well. Both preparations appear to contain little or no functional mitochondrial membranes (11, 21). Protein was determined by the method of Bradford (6).

Measurement of Membrane Potential. Aliquots of cotton or radish vesicles were incubated in a buffered, osmotically adjusted medium containing KSCN[14]CNS as described by Hassidim et al. (11) with or without 5 mm Tris-ATP or 0.5 to 1.0 mm Na3Fe(CN)6. At the end of the uptake period (usually 5–10 min), the medium was diluted, rapidly filtered, and radioactivity remaining on the washed filter determined in a scintillation counter. All treatments were performed in triplicate with the SD averaging ±10% of the mean. The data presented are from representative experiments repeated at least three times.

Determination of Oxidoreductase Activity. Assays were carried out at room temperature in 1 ml cuvettes containing 0.4 M sucrose, 3 mm MgSO4, 0.5% (w/v) BSA, 10 mm BIP-Mes2 (pH 5.5), 0.1 mm NADH, and 0.5 mm Na3Fe(CN)6. The reaction was initiated by the addition of 10 μl of cotton or radish vesicles (approximately 20 μg and 40 μg protein, respectively). A reference cuvette containing the same solution but without the vesicles was used to correct for the chemical oxidation of NADH by

Abbreviations: BTP, bis-tris propane (1,3 bis [tris hydroxymethyl]-methylaminol)-propane; DCIP, 2,6-dichlorophenol-indophenol; DQ, duroquinone (tetramethyl-p-benzoquione); FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; NEM, N-ethylmaleimide; PCMB, p-chloromercuribenzoic acid; m.p., membrane potential; SCN-, thiocyanate.

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ferricyanide. Oxidation of NADH and reduction of ferricyanide was observed by following loss of absorbance at 340 and 420 nm, respectively, in a Bausch and Lomb Spectronic 2000 dual beam spectrophotometer. Cyt c reduction was measured at 550 nm and DPIP at 600 nm. Because of low NAD(P)H oxidase activity, rates of NAD(P)H oxidation in the presence of these acceptors were estimated by assuming a NAD(P)H oxidized/Cyt c reduced ratio of 0.5 and a NAD(P)H oxidized/DPIP reduced ratio of 1.0. Data presented are the means of at least two separate determinations for a representative experiment.

Chemicals. KSCN[14C]N was obtained from Radiochemical Center, Amersham (U.K.). NADTP was obtained from Boehringer (Mannhein, W. Germany) and was converted to Tris-ATP by passage through a Tris-Dowex 50 column. All other chemicals were from Sigma Chemical Co. (Tel Aviv, Israel).

RESULTS AND DISCUSSION

Evidence that a membrane potential (m.p.) can be generated in a plasmalemma-enriched vesicle fraction using energy associated with redox activity is presented in Table I. When cotton roots are homogenized in a medium containing 100 mM ascorbate, addition of either ATP or ferricyanide leads to an increase in amount of SCN⁻ taken up, indicating formation of a m.p. inside positive. The effect is not observed when the Na salt of ferricyanide (the reduced form) is substituted for the Na salt of ferricyanide. This suggests that SCN⁻ uptake is not due to the generation of a diffusion potential by a mobile penetrating cation in the presence of an immobile anion, but is due to a redox reaction. While FCCP at 10 μM might inhibit electron flow, the generally accepted uncoupling action of FCCP (Table I) makes it likely that the ferricyanide-induced m.p. results from an accumulation of H⁺. No other ions are added to the medium which could contribute to the m.p. and addition of KCl was without effect. The effects of ATP and of ferricyanide on SCN⁻ accumulation when both are supplied together are approximately additive (data not shown). This may indicate that ATP and ferricyanide affect different systems in the same vesicle and/or that ATP and ferricyanide act on different populations of vesicles; for example, inside-out versus right-side-out vesicles.

When cotton roots are homogenized in a medium without ascorbate, the size of the m.p. observed in the presence of ATP is reduced, presumably due to the absence of the reducing agent, though it is still detectable. The ferricyanide-induced m.p., however, is no longer observed. Even without direct measurements of redox activity, the data in Table I suggest the following model: electrons are transported across the membrane by a carrier system from ascorbate within the vesicle to ferricyanide in the medium, while H⁺ remain inside the vesicle. This electrogenic transmembrane electron transport results in formation of a m.p., inside positive.

A similar model is proposed by Rubinstein and Stern (22) based on direct measurements using root segments, except that a reduced pyridine nucleotide is the electron donor. A tendency toward a more positive m.p. was also observed when intact cells were exposed to ferricyanide (13, 24). The presumed mixed orientation of the vesicles makes it impossible to determine if the direction of electron transport across the membrane depends only on the redox potential gradient or if there is a preferential direction.

In what may be a different redox system, Morré et al. (18) suggest that external NADH is oxidized by a monodehydroascorbate produced within soybean plasmalemma vesicles. If this is occurring in cotton vesicles, the process does not lead to a positive m.p., since NADH alone has no effect on SCN⁻ uptake by cotton vesicles homogenized in ascorbate (Table I). Furthermore, exogenous NADH cannot substitute for ascorbate within the vesicle as the electron donor to ferricyanide responsible for generating an inside positive m.p. (Table I); this is true even though the vesicle membranes can mediate the transfer of electrons from NADH in the medium of ferricyanide (Table III).

The failure to observe a stimulation of SCN⁻ uptake with ferricyanide plus NADH using vesicles homogenized in a medium containing ascorbate is probably due to reduction of ferricyanide preferentially by the exogenous NADH rather than by the ascorbate within the vesicle; this conclusion is based on the observation that SCN⁻ uptake is stimulated if 2 mM instead of 1 mM ferricyanide is added together with the NADH (data not shown). Similar observations to those presented above were made using radish vesicles, although the amount of SCN⁻ accumulated in the presence of ferricyanide is less by approximately one-half when expressed per unit membrane protein.

Figure 1 shows the time course of SCN⁻ accumulation by radish vesicles in three concentrations of ferricyanide. The amount of SCN⁻ associated with the vesicles is optimal after 10

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Table 1. Effect of Ascorbate in the Grinding Medium on SCN⁻ Uptake by Plasmalemma-Enriched Vesicles from Cotton Roots

<table>
<thead>
<tr>
<th>Additions</th>
<th>SCN⁻ Uptake (+ Ascorbate)</th>
<th>SCN⁻ Uptake (− Ascorbate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol-mg⁻¹ protein-min⁻¹</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>5 mM Tris-ATP</td>
<td>10.4</td>
<td>5.2</td>
</tr>
<tr>
<td>1 mM Na₅Fe(CN)₉</td>
<td>21.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1 mM Na₅Fe(CN)₉</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Na₅Fe(CN)₉ + 10 μM FCCP</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>1 mM NADH</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Na₅Fe(CN)₉ + NADH</td>
<td>2.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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FIG. 1. Time course of SCN⁻ uptake by plasmalemma-enriched vesicles from germinating radish seeds in the presence of three concentrations of ferricyanide. Radish seeds were imbibed on distilled H₂O for 24 h and vesicles isolated by the methods of Rasi-Caldogno et al. (21) except that 100 mM ascorbate, but not β-mercaptoethanol and ATP, was present in the grinding medium. The vesicles were incubated with [14C]SCN⁻ in a medium containing the substances described in the caption to Table I and including 0 mM (○), 0.1 mM (○), 0.5 mM (●), or 1.0 mM (□) Na₅Fe(CN)₉. Aliquots were removed at the times indicated and rapidly filtered to determine SCN⁻ uptake. Data represents means of triplicates which did not vary more than about 10% from the mean.
Table II. Effect of Various Inhibitors on Ferricyanide-Induced SCN⁻ Uptake into Plasmalemma-Enriched Cotton Vesicles

Cotton roots were homogenized in a medium containing 100 mM ascorbate, and the purified vesicles incubated as described in the caption to Table I. Additions were made to the incubation medium just prior to the start of the 10-min uptake period. Data are means of triplicates which did not vary more than about 10% from the mean.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SCN⁻ Uptake</th>
<th>Change from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol mg⁻¹ protein-min⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>500 μM Na₃Fe(CN)₆</td>
<td>10.1</td>
<td>-20</td>
</tr>
<tr>
<td>+150 μM Vanadate</td>
<td>8.0</td>
<td>-11</td>
</tr>
<tr>
<td>+1 mM PCMB</td>
<td>7.6</td>
<td>-11</td>
</tr>
<tr>
<td>+1 mM NEM</td>
<td>9.0</td>
<td>-11</td>
</tr>
<tr>
<td>+50 μg oligomycin·mg⁻¹ protein</td>
<td>10.6</td>
<td>+5</td>
</tr>
<tr>
<td>+500 μM NaN₃</td>
<td>0.9</td>
<td>-91</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>7.3</td>
<td>-100</td>
</tr>
<tr>
<td>+500 μM NaN₃</td>
<td>0</td>
<td>-100</td>
</tr>
</tbody>
</table>

Table III. Effect of Various Acceptors on NAD(P)H Oxidation by Plasmalemma-Enriched Radish Vesicles

Vesicles were isolated by the methods of Rasi-Caldogno et al. (21) except that 100 mM ascorbate but not β-mercaptoethanol and ATP was present in the grinding medium. The oxidation of NAD(P)H or reduction of Cyt c or DCIP was determined spectrophotometrically as indicated in "Materials and Methods." Oxidation of NaN₃ and NADPH alone was 1.28 and 0.4 nmol·mg⁻¹ protein·s⁻¹, respectively. Data represent means of replicates for a representative experiment.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Fe(CN)₆²⁻</th>
<th>Cyt c</th>
<th>DQ</th>
<th>DCIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>5.5</td>
<td>0.36</td>
<td>1.3</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>1.3</td>
<td>0.03</td>
<td>1.3</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>

min and does not greatly decrease thereafter. In another experiment (not shown), equimolar ascorbate was added to the vesicles 5 min after addition of ferricyanide, so that all of the remaining ferricyanide was immediately reduced. This resulted in no further accumulation of SCN⁻ beyond the 15 pmol mg⁻¹ protein already present by that time. When an additional 0.7 mM ferricyanide was added 20 min later, the accumulation of SCN⁻ resumed, and after a further 5 min, approached the level observed in vesicles which had been incubated in ferricyanide alone during the same period (22 versus 28 pmol mg⁻¹ protein, respectively). These data indicate that the vesicles were not leaky and that they retained their ability to generate a m.p. by redox activity over a 30 min period.

Uptake of SCN⁻ into both cotton and radish vesicles was determined after 10 min for ferricyanide concentrations of 0.066 mM to 1 mM (data not shown). The results support those presented in Figure 1—the concentration of ferricyanide giving half-maximal SCN⁻ accumulation is approximately 0.2 mM. This concentration is similar to the one observed for half-maximal stimulation of redox activity (20), depolarization of the m.p. (13), and inhibition of ion uptake (22) in a variety of tissue and cell systems.

Attempts were made to inhibit formation of the ferricyanide-induced m.p. in cotton vesicles. As shown in Table II, only NaN₃ had any striking effect, but because the accumulation of SCN⁻ in the presence of ATP was also eliminated by NaN₃, the effect is likely to have resulted from dissipation of the m.p. by the permeating N₃⁻ anion. Since the vesicles were not preincubated in the inhibitors, it is possible that more striking effects would have occurred later. It is difficult to compare the results of various inhibitors added in vivo (e.g., 1, 10, 13, 23) with the vesicle system presented here because of indirect effects which may occur when intact cells are used.

The following compounds were added as possible substitutes for ferricyanide to generate a m.p. in ascorbate-containing cotton or radish vesicles: duroquinone (200 μM), Cyt c (250 μg/ml), dehydroascorbic acid (1 mM), DCIP (50 μM). None of these electron acceptors increased the levels of SCN⁻ accumulations above that of the control (data not shown). Ferricyanide specificity was also observed for the redox-induced inhibitions of K⁺ uptake in corn roots (22). However, DCIP does mimic ferricyanide in depolarizing the m.p. of Phaseolus (24) and Lemma (13), but the interpretation of this effect may be complicated by the permeation of DCIP.

Besides the ability to generate a m.p. by transmembrane redox activity in the presence of ferricyanide, the vesicle preparations also possess a putative electron transport activity in the plane of the membrane. Evidence for this is NAD(P)H:ferricyanide oxidoreductase activity (Table III). Table III shows further that ferricyanide oxidizes NADH at only one-fourth the rate of NaN₃. Oxidation of NaN₃ by vesicles in the presence of ferricyanide is six times more rapid than in its absence, and the concentration of ferricyanide resulting in a half-maximal rate of NaN₃ oxidation is between 0.1 and 0.2 mM (data not shown). However, because of the low extinction coefficient for ferricyanide, the spectrophotometric method used is not sensitive enough to detect reduction of this agent when added alone.

Other electron acceptors (Cyt c, DCIP, and DQ) stimulate the oxidation of NAD(P)H in the presence of the membranes, although none is as active as ferricyanide (Table III). Qualitatively similar results for the relative rate of oxidation of NADH by ferricyanide, Cyt c and DCIP, were obtained by Barr et al. (3) and Buckhout and Hrubec (7) using right-side out plasmalemma vesicles from soybean hypocotyls and maize roots, respectively. NADH:DQ oxidoreductase activity in plasmalemma-enriched microsomes isolated from corn coleoptiles has been reported by DeLuca et al. (9).

It is interesting that compounds other than ferricyanide are reduced by NADH (Table III) in the presence of vesicles, presumably along the plane of the membrane, but these same compounds are unable to stimulate transmembrane electron transport as measured indirectly by generation of a m.p. in ascorbate-loaded vesicles. In part, this distinction is probably due to the methods used to detect the two types of redox activity. Thus, Cyt c oxidizes NaN₃ at a rate less than one-tenth that of ferricyanide, a rate which could not be detected easily measuring transmembrane electron transport by SCN⁻ uptake (Table I). However, DQ is one-fourth as active as ferricyanide in oxidizing NADH, yet has no effect on SCN⁻ uptake. We would be able to...
detect a stimulation of SCN\(^-\) uptake (i.e. of transmembrane electron transport) if DQ were one-fourth as active as ferricyanide.

The possibility exists, therefore, that transport of reducing equivalents in the plane of the membrane represents a system separate from transmembrane electron transport and is mediated, at least in part, by different carriers. Because of differences in relative redox potential, the carrier complex of the transmembrane system reduces ferricyanide but not DQ, while the complex associated with transport in the plane of the membrane reduces a wider range of compounds. If this is true, it may eventually be possible to separate complexes belonging to the two systems on the basis of the substances they reduce.

Acknowledgments—We are grateful for the technical assistance of Ms. Dorit Gabay and for helpful suggestions of Dr. A. I. Stern (University of Massachusetts, Amherst).

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