Electrogenic Proton Translocation by the ATPase of Sugarcane Vacuoles

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ABSTRACT

Existence of a proton-translocating ATPase on the tonoplast of higher plants has been further confirmed by use of two experimental systems: (a) intact isolated vacuoles from sugarcane cells and (b) vesicles prepared from the same source. Addition of MgATP to vacuoles polarized the tonoplast by 40 millivolts to a value of +20 millivolts, but a large preexisting pH gradient across the membrane restricted the pH change to 0.2 unit. In vesicle preparations, the tonoplast was polarized to +66 millivolts by the addition of MgATP and the intravesicular space was acidified by 1 pH unit to pH 5.5. Proton translocation equilibrium is controlled by the protonotive potential difference, maximal at 125 millivolts for sugarcane cells. Energization of the tonoplast occurred at physiological concentrations of MgATP. specificity of MgATP for proton translocation was indicated by a much smaller effect of MgADP and MgGDP on the electrochemical gradient, although these substrates were also hydrolyzed by tonoplast preparation.

In recent years, availability of better methods of isolating intact functional vacuoles from higher plants has permitted better insight into the physiology and biochemistry of this organelle (3, 20, 25). A tonoplast-bound ATPase which functions in energizing the membrane exists in higher plants (4, 24, 26). The evidence for this phenomenon comes predominantly from small fungal vacuoles (10), lysosome-like membrane vesicles (13), and microsomal membrane fractions (3, 6, 19). Evidence from intact, large-sized, higher plant vacuoles is less abundant. Wagner and Lin (26) reported that ATP added to tulip vacuoles caused an acidification of the intravesicular space and a slight polarization of the membrane potential. Miller et al. (15) noted a small depolarization of the membrane potential of red beet vacuoles in the presence of ATP. These findings are in agreement with widespread observations that plant vacuoles, in situ, have an acidic interior (9) and possess a positive membrane potential oriented toward the cytoplasm (2).

Intact vacuoles can be isolated from sugarcane cells grown in suspension cultures. These isolated vacuoles hydrolyze ATP (23, 24) and transport sugars against a concentration gradient (22). Addition of MgATP to isolated sugarcane vacuoles polarized the membrane potential towards a more positive value, but the voltage when measured as SCN⁻ accumulation was significantly less (25 mv) than had been measured for vacuoles in situ (60

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MATERIALS AND METHODS

Plant Material and Vacuole Isolation. Sugarcane cell suspensions (a subclone of Saccharum sp. hybrid H50-7209) were grown in White’s inorganic salt mixture supplemented with yeast extract, arginine, sucrose, vitamins, and 2,4-D (17). The cells, harvested 9 d after subculture, constituted 3 to 4 g fresh weight/100 ml medium. Vacuoles were isolated from protoplasts by centrifugation on a Ficoll cushion (21). Tonoplast vesicles were prepared by breaking vacuoles in a glass homogenizer (about 40 strokes) in a large volume of medium containing 25 mM Tricine-Mes (pH 6.5), 10 mM MgSO₄, and 250 mM mannitol. The membrane suspension was layered over 10% dextran T70 in homogenization buffer and centrifuged at 100,000g for 60 min. The membrane fraction used for experiments sedimented in a layer above the dextran. The volumes of vacuoles and tonoplast vesicles were calculated from the water-permeable but dextran-impermeable space.

Membrane Potential Measurements. Absorption changes of oxanol VI² and di O-C₆(3) were used to measure membrane potential (1). Vacuoles or tonoplast suspension was added to a solution containing 25 mM Tricine-Mes (pH 6.5), 10 mM MgSO₄, 4 µM of the optical probe, and 500 mM (vacuoles) or 250 mM mannitol (tonoplasts). The change in absorbance at 603 and 580 nm for oxanol VI and 500 and 480 nm for di O-C₆(3) was followed on a dual wavelength spectrophotometer. Artificially imposed K⁺ gradients in the presence of 10 µg ml⁻¹ valinomycin were used to calibrate the membrane potential. Membrane potential was calculated using the change in absorbance from the time immediately after the initial shift caused by addition of MgATP until the steady state value is reached. Potassium diffusion potential was calculated according to the Nernst equation. Potassium concentration was measured using a specific ion electrode.

pH Measurements. The internal pH of vacuoles and tonoplast

²Abbreviations: oxanol VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentomethyle oxanol; di O-C₆(3), 3,3’-dipentylxocarbocyanine iodide; FCCP, p-trifluoromethoxy carbonyl cyanide.
vesicles was measured as the fluorescence quenching of the permeant amines quinacrine and acridine orange (12). Vacuoles or tonoplast vesicles were suspended in 25 mM Tricine-Mes (pH 6.5), 10 mM MgSO4, 5 mM KCl, and either 500 mM (vacuoles) or 250 mM mannitol (tonoplasts). Either 10 µM quinacrine or 5 µM acridine orange was added and changes in fluorescent emission were measured at 425 → 505 nm (excitation → emission) for quinacrine or 493 → 530 nm for acridine orange. The pH gradient was calculated from the fluorescence quench and the volume of vacuoles or tonoplast vesicles.

Chemicals. Oxanol VI and di O-C6(3) were gifts from G. Hauska (Regensburg, West Germany). Fluorescent amines were from Sigma Chemical and nigericin and valinomycin were from Calbiochem.

RESULTS

Membrane Potential of Isolated Vacuoles. Isolated vacuoles of sugarcane have a negative inside membrane potential when measured as the accumulation of tetracyanophenonium ion (11), but the value is close to zero when measured as the accumulation of thiocyanate (24). Both values must be regarded with caution since it is difficult to correct for a significant amount of probe adsorption. Furthermore, each of these probes is applicable only if the membrane potential is significantly greater than zero in either direction. Optical probes were substituted in this study to assess the reliability of the previous measurements and to follow the effects of nucleotides and uncouplers. Oxanol VI gave the largest response. Addition of valinomycin to vacuoles in the ‘resting state,’ i.e. without addition of MgATP, changed the oxanol VI absorption only slightly. Therefore, the membrane potential in the resting state is very close to the potassium diffusion potential which was 26 mv (Table I). The internal concentration of potassium in these vacuoles was 10.5 mM. The addition of the uncoupler FCCP caused additional polarization of the tonoplast to -79 mv (Table I)—an expected consequence if the gradient of 1.4 pH units was abolished.

The addition of MgATP caused a rapid decrease of oxanol VI absorption followed by a new steady state after 2 min (Fig. 1, plot A). This change corresponded to about a 40 mv positive polarization of the membrane potential. In the presence of FCCP, the MgATP-dependent polarization of the membrane potential was much slower and smaller (Fig. 1, plot B). Valinomycin and nigericin completely inhibited the response (Fig. 1, plot C). MgADP addition caused only a small change of the membrane potential (Fig. 1, plot D; Table I).

When 4 µM of the carbocyanine dye, di O-C6(3), was used to measure membrane potential, results paralleled those obtained with oxanol VI. The changes in absorbance were smaller but the calibration of the response showed the same MgATP-catalyzed polarization of the membrane potential (Table I).

When the ‘resting potential’ of vacuoles was decreased to -10 mv by addition of 100 mM KCl, MgATP-dependent polarization was reduced to 34 mv (Fig. 2). The plateau level of the membrane potential was, therefore, the same as in the control, i.e. without additional K+. The addition of K+ after MgATP-induced polarization had no further effect on the membrane potential (Fig. 3). The sensitivity of the membrane potential to FCCP and to valinomycin plus nigericin suggests that the positive polarization catalyzed by MgATP is caused by proton translocation.

Membrane Potential of Tonoplast Vesicles. In the presence of MgATP, the membrane potential of intact vacuoles was positive, but the value was much lower than values reported for microsomal fractions (3). Tonoplast vesicles derived from sugarcane vacuoles exhibited a greater response to MgATP than did intact vacuoles. The membrane potential change was more than 60 mv (Table I) and was completely reversed by valinomycin plus nigericin (Fig. 4). This quantitative difference between vacuoles and tonoplast vesicles may be due either to the efflux of easily permeable intravacuolar cations acting to counterbalance the MgATP-catalyzed cation influx and thereby reducing the degree of polarization, or to the larger pH gradient in intact vacuoles, which, for thermodynamic reasons, stalls the membrane potential at a lower value.

pH Gradient of Isolated Vacuoles. Isolated vacuoles retained an acid interior (11). Addition of vacuoles to a solution of the permeant amine quinacrine caused a fluorescence quench which indicated a pH gradient of 1.4 units (Table II). The addition of MgATP caused a very slight, barely detectable acidification of 0.2 pH unit (Table II). Addition of valinomycin plus nigericin collapsed the pH gradient and reversed the fluorescence quench. It is difficult to reconcile this small acidification of the intravacuolar space with the concept of proton translocation by the tonoplast-bound ATPase. However, it is possible that either the preexisting pH gradient does not allow further acidification, or the buffering capacity of the vacuolar solute makes it difficult to detect a pH change. These possibilities were tested with tonoplast vesicles from which intravacuolar solutes were removed during their preparation.

pH Gradient of Tonoplast Vesicles. A much more vivid effect of MgATP-dependent quenching of both acridine orange (Fig. 5) and quinacrine (Fig. 6) fluorescence was obtained when tonoplast vesicles were used. The pH gradient catalyzed by MgATP was 0.9 to 1.2 units (Table II). The addition of valinomycin plus nigericin collapsed the pH gradient and reversed the fluorescence quenching (Figs. 5 and 6). When valinomycin plus nigericin was added before the addition of MgATP, the acidification was

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Probe</th>
<th>Addition</th>
<th>Final Membrane Potential</th>
<th>Change of Membrane Potential by Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuoles</td>
<td>K+</td>
<td></td>
<td>-26 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oxanol VI</td>
<td></td>
<td>-23 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oxanol VI</td>
<td>FCCP</td>
<td>-79 ± 20</td>
<td>-54 ± 28</td>
</tr>
<tr>
<td></td>
<td>oxanol VI</td>
<td>MgATP</td>
<td>+19 ± 12</td>
<td>+42 ± 19</td>
</tr>
<tr>
<td></td>
<td>di O-C6(3)</td>
<td>MgATP</td>
<td>+14 ± 7</td>
<td>+39 ± 19</td>
</tr>
<tr>
<td></td>
<td>oxanol VI</td>
<td>MgADP</td>
<td>-14 ± 6</td>
<td>+7 ± 5</td>
</tr>
<tr>
<td>Tonoplast vesicles</td>
<td>oxanol VI</td>
<td>MgATP</td>
<td>+66 ± 5</td>
<td>+66 ± 5</td>
</tr>
</tbody>
</table>
(b) the ATP-catalyzed ATP-energized proton translocation is not different. In the absence of MgATP or MgGDP, valinomycin and nigericin, 5 μM each, inhibited. Valinomycin alone did not prevent the MgATP-dependent acidification (Fig. 6), but the subsequent addition of nigericin not only prevented acidification but collapsed the pH gradient. MgADP and MgGDP had no effect on the internal pH of tonoplast vesicles (Fig. 6; Table II). Results from these experiments confirm previous findings (11, 24) that the tonoplast-bound ATPase of sugarcane catalyzes electrogenic proton translocation.

**DISCUSSION**

The membrane potential of isolated sugarcane vacuoles was in the negative range (−20 to −30 mv), similar to the range in lutoids (7) and sugar beet vacuoles (8, 15). The addition of MgATP shifted the membrane potential toward the more positive direction by 40 to 50 mv so that it was in the range of 20 mv. In lutoids and sugar beet, the membrane potential remained negative with the addition of ATP (7, 8, 15), and in tulip vacuoles the ATP-catalyzed polarization was only 2 to 5 mv (26). This difference may arise either from (a) different techniques of measurement, (b) different physiological conditions of isolated vacuoles, (c) different plant material, or (d) different permeable intravacuolar content. Reports on chloride stimulation (3) and nigericin stimulation (20) of ATPase suggest that the vacuolar ATPase is controlled by the proton potential so that the emphasis of the protonic force shifts between the membrane potential and the pH gradient. This phenomenon has been known since the protonmotive force was first found in chloroplasts and mitochondria (16). A preexisting pH gradient in the deenergized vacuoles might prevent a strong polarization by the ATPase. Release of internal solutes and consequent dissipation of the pH gradient during the preparation of tonoplast vesicles from sugarcane vacuoles led to a larger MgATP-catalyzed positive polarization of the membrane potential. This change of the membrane potential (60–70 mv) is comparable to the published values for other tonoplast vesicles (3, 13).

ATP-dependent acidification of the intravacuolar and intravesicular space has also been reported in red beet (4), oat root (6), corn coleoptile (14), and tulip petals (26). In sugarcane, however, very little MgATP effect was noted in isolated vacuoles, while in tonoplast vesicles MgATP elicited greater acidification. This difference was due not only to the greater surface/volume ratio of vesicles, but also to the thermodynamic force of the proton potential of intact vacuoles. In the presence of MgATP, isolated vacuoles have an electrochemical gradient of 115 mv and tonoplast vesicles of 125 mv. While this is a small difference, the pH gradient is larger in vacuoles; it was 1.5 pH units even in the nonenergized state. The control of the energization by the
protonmotive potential difference was also seen in experiments where the membrane potential was shifted by addition of K⁺ to the medium. With the initial membrane potential depolarized from -26 to -10 mV, the plateau value of the MgATP-catalyzed membrane potential was similar to control conditions. There was considerable GDP and ADP hydrolytic activity on the tonoplast, but these nucleotides had little or no effect on the membrane potential and pH gradient. This supports previous
PROTON TRANSLOCATION IN SUGARCANE VACUOLES

Table II. Internal pH of Sugarcane Vacuoles and Tonoplast Vesicles

Internal pH was determined as described in “Materials and Methods.” MgATP and MgADP were added to give a final concentration of 3 mM. Values are the mean of at least three determinations ± SD.

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>Probe</th>
<th>Addition</th>
<th>pH</th>
<th>Change of pH by Addition</th>
<th>pH Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuoles</td>
<td>quinacrine</td>
<td>MgATP</td>
<td>5.09 ± 0.28</td>
<td>0.22 ± 0.08</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>quinacrine</td>
<td>MgADP</td>
<td>4.87 ± 0.18</td>
<td>1.23 ± 0.30</td>
<td>1.63</td>
</tr>
<tr>
<td>Tonoplast vesicles</td>
<td>quinacrine</td>
<td>MgATP</td>
<td>6.5</td>
<td>0.22 ± 0.23</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>quinacrine</td>
<td>MgADP</td>
<td>5.27 ± 0.30</td>
<td>1.23 ± 0.30</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>acridine orange</td>
<td>MgATP</td>
<td>5.61 ± 0.27</td>
<td>0.89 ± 0.27</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>quinacrine</td>
<td>MgADP</td>
<td>6.28 ± 0.23</td>
<td>0.89 ± 0.27</td>
<td>0.89</td>
</tr>
</tbody>
</table>

findings that the proton translocator is specific for MgATP (24) and confirms that GDPase and ADPase do not participate in formation of the electrochemical gradient. The ATP-dependent positive polarization of the membrane potential and the acidification of the internal space of both intact vacuoles and tonoplast vesicles confirm previous suggestions that the ATPase of vacuoles is a proton-translocating device.

Acknowledgments—We would like to thank Professor G. Hauska and Dr. E. Hurt (Universität Regensburg) for the membrane potential probes and for the use of the Amico-Chance dual wavelength spectrophotometer and Dr. H. Yamamoto (University of Hawaii, Honolulu) for the use of the Perkin-Elmer dual wavelength spectrophotometer.

LITERATURE CITED


Fig. 5. Fluorescence quenching of acridine orange by tonoplast vesicles. Tonoplast vesicles (30 μl internal volume, 300 μg protein) were suspended in 1.5 ml 25 mM Tricine-Mes (pH 6.5), 10 mM MgSO4, 5 mM KCl, 250 mM mannitol, and 5 μM acridine orange at pH 6.5. Final concentration of MgATP was 3 mM; valinomycin, 5 μM; nigericin, 5 μM.

Fig. 6. Fluorescence quenching of quinacrine by tonoplast vesicles. Tonoplast vesicles (20 μl internal volume, 200 μg protein) were suspended in buffer as described in Figure 5. Final concentration of quinacrine was 10 μM.


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