H⁺ Efflux and Hexose Transport under Imposed Energy Status in Maize Root Tips

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ABSTRACT

The relationship between changes in H⁺ flux and sugar transport in maize Zea mays L. DEA root tips have been investigated using two methods for controlling the cellular nucleotide level: (a) incubation in the presence of a glucose analog, the 2-deoxyglucose, which decreased the ATP level to less than 15% of its initial value within 60 minutes without changing the ADP and AMP levels; (b) an hypoxic treatment which also decreased the ATP level but with a concomitant rise in ADP and AMP. In both cases the rate of hexose transport was not modified until ATP had dropped to 70% of its initial value; then it decreased with the cellular ATP level. The residual uptake rate at very low ATP concentrations still represented 50% of the maximum rate with the dGlc treatment but only the diffusion rate in anoxia. H⁺ efflux was abolished in anoxia but not by the 2-deoxyglucose treatment, in spite of a lower cellular ATP concentration. Our results are consistent with an inhibition of H⁺-ATPase activity in anoxia by the high levels of cellular ADP and AMP, and provide in vivo evidence that sugar uptake is dependent upon the proton motive force rather than cellular ATP concentration. The absence of stimulation of H⁺ extrusion by ferricyanide in either normoxic or hypoxic conditions suggests that a redox system does not appear to contribute to H⁺ secretion under the conditions of this investigation.

When plant roots are exposed to anoxic conditions, a rapid and reversible K⁺ efflux occurs as a consequence of membrane depolarization rather than of an increase in permeability (7). Such a depolarization could induce a blockage of the secondary transport system driven by protons (20). The depolarization of anoxic cells is often attributed to an insufficient energy supply. This assessment is supported by data showing some correlations between membrane potential and ATP level modulated by low oxygen pressure (13), by inhibitors of energy metabolism (19, 27) or imposed by perfusion techniques as in giant alga cells (14). However, these authors noticed a number of circumstances in which this correlation does not hold. Lőpért's data (9) obtained in Lemna at different oxygen partial pressures would suggest that there is no correlation between ATP level and membrane potential, whereas Mimura et al. (15) show that ADP and AMP, which increase under anoxia, are competitive inhibitors of plasma-lemma ATPase. These findings suggest that factors other than ATP are implicated in the regulation of membrane potential under anoxic conditions. It is well established that sugar or amino acid transport in plant cells is driven by pmf¹ and that ATP fuels this electrogenic process (1, 6, 20, 27), but it is difficult to find concurrent in vivo data on cellular free energy change, pmf generation, and substrate transport. Furthermore, the concept that ATP is the only fuel of these processes has been questioned by several reports on the possible involvement of another H⁺ secreting system linked to a redox chain with NAD(P)H as electron donor and stimulated by ferricyanide (4, 16, 21). Thus, the aim of the study reported here was to examine changes in energy charge, pmf, and substrate transport in a single experimental system.

In this paper, the H⁺ extrusion and sugar transport in maize root tips have been investigated using dGlc and pO₂ for controlling the cellular nucleotide level and FC for modulating the activity of the H⁺-pump.

MATERIALS AND METHODS

Chemicals

All the unlabeled chemicals were purchased from Sigma Chemical Co. and were of the highest available purity. d-(U-¹⁴C)glucose (11 GBq/mmol) was from Commissariat à l’Energie Atomique, France; d-(U-¹⁴C)-fructose (11 GBq/mmol) was from Radiochemical Center, Amersham, England; 3-O-methyl-d-(U-¹⁴C)glucose (11.7 GBq/mmol); and 2-deoxy-d-(U-¹⁴C)glucose (2.15 GBq/mmol) were from New England Nuclear.

Plant Material

Maize seeds (Zea mays L. DEA, Pioneer) were germinated in the dark at 25°C on wet filter paper. At 3 d after imbibition, 3 mm primary root tips (2.2 mg fresh weight each) were excised and incubated for 4 h at 25°C in a mineral solution (22), aerated by air bubbling, and buffered by 5 mM MOPS adjusted to pH 6.2 by KOH. In some experiments, the young 3 d seedlings were previously acclimated to anoxia with 18 h incubation period in hypoxia (2-4 kPa O₂) as described (24).

Transport Studies

Root tips were placed in groups of 20 in disposable syringes (5 mL) containing 1.9 mL of the above medium. The syringes

¹ Abbreviations: pmf, proton motive force; dGlc, 2-deoxy-d-glucose; pO₂, oxygen partial pressure; MOPS, 3-(morpholino)propanesulfonic acid; 3-OMG, 3-O-methyl-d-glucose; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; FC, fusicoccin; DES, diethylstilbestrol; EB, erythrosin B.
were fitted with 12 × 0.45 mm needles on vacuum rubber tubes flushed with air or with mixtures of N₂ and O₂. Having equilibrated at 25°C, sugar uptake was initiated by addition of 100 μL of radioactive substrate. After 5 to 15 min, during which the uptake was linear in all conditions tested, the uptake was terminated by washing the tips five times for 1 min with 20 mL of ice-cold MOPS solution. The ice-cold tips were placed in groups of 10 in 2 mL of aqueous scintillation fluid (ACS II, Amersham Corp.) and counted. All the determinations were done in duplicate.

Nucleotide Determination

Aerobically treated root tips were frozen in liquid nitrogen. For hypoxic treatments, root tips were placed in groups of 10 in polyethylene tubes containing 2 mL of medium and fitted with rubber caps. Hypodermic needles through the rubber caps were used to gas the solution and headspace, one needle bubbling gas into the solution, the other giving gas exit from the tube. After the desired time of incubation, the solution was forced out from each tube by inverting the gas connections, and the tube was placed in liquid nitrogen to quickly freeze the roots. The frozen tubes were stored at −30°C and the nucleotides were extracted for ATP, ADP, and AMP estimation using published procedures (22, 24).

Control of ATP Levels by dGlc

The washed root tips were preincubated in a fresh medium containing 50 mM dGlc. After appropriate times they were rinsed with dGlc-free medium for 5 min and used for transport or proton-exit studies and nucleotide determinations.

Measurement of H⁺ Fluxes

Proton extrusion or uptake was measured at 25°C with a glass pH electrode (Ingold Fektrode 403 M8) fitted into a 5 mL water-jacketed vessel containing 3 mL medium and a small magnetic stirrer. Gas mixtures of known compositions could be bubbled in the medium when desired. The signal was used as a function of time.

For proton extrusion, a medium containing 2 mM CaCl₂, 1 mM KOH, and either 3 or 9 mM KCl was bubbled with water-saturated gas mixtures (N₂/O₂) containing 5 kPa CO₂, in order to buffer the medium and prevent acidification by respiratory CO₂. After pH stabilization (at about 5.8), 60 root tips were added. Another short equilibrium time was needed before reaching the basal acidification rate of the medium. Then, 10 to 20 μL of the desired reagent were injected. The electrode response was recorded for 10 to 20 min and calibrated at the end of each experiment by addition of 50 to 100 nM of HCl or NaOH. In the ferricyanide experiments, K₂Fe(CN)₆ had previously been transformed into the BTP salt according to Marré et al. (10). This was to avoid any change in the K⁺ concentration of the incubation medium which contained 0.5 mM Mes/BTP, 0.5 mM CaSO₄, and which was bubbled by gas mixtures as above. Initial pH was adjusted to 6.00 with BTP.

For sugar-coupled H⁺ uptake studies, the medium which contained 2 mM CaCl₂ and 2 mM KCl was not bubbled by the gas. After adding the root tips, it took about 10 min for the medium to equilibrate from pH 5.8 to pH 5.6. Then the sugars were added. The response was calibrated by adding 10 to 20 nM of HCl to the medium.

RESULTS

Effect of dGlc on Adenine Nucleotide and Sugar Transport

When the sugar-depleted root tips were incubated in 50 mM dGlc, the cellular ATP level declined sharply to less than 15% of the control within 60 min, and then became stable (Fig. 1). During this time, the ADP and AMP levels remained stable (Table I). This process was reversible, but it took at least 30 min (after the transfer of the root tips to the dGlc-free medium) before the ATP level rose again. Addition of glucose increased the rate of the recovery process (Fig. 1) and the root tips were able to resume growth at a normal rate (data not shown).

The effect of dGlc on the rate of glucose uptake was studied in a parallel experiment (Fig. 1). Incubation for 20 min in 50 mM dGlc, which induced a 60% decrease of ATP, had little effect on the rate of 2 mM glucose uptake. After longer incubation in dGlc, the ATP level became very low, but the residual uptake rate remained higher than 50% of its initial

![Figure 1. Effect of dGlc on the cellular ATP level and glucose uptake. ATP (△) and 2 mM glucose uptake (●) in dGlc-free medium were assayed in excised maize root tips after various incubation times in 50 mM dGlc. After 45 min in dGlc as indicated by the arrows, some root tips were rinsed with MOPS buffer for 5 min and then transferred to dGlc free medium (open symbols) supplemented or not with 100 mM glucose. ATP (△, ○) and glucose uptake (○, □) were measured in order to check the reversibility of the dGlc treatment. The 100% values were 1306 pmol-tip⁻¹ for ATP and 220 pmol-tip⁻¹-min⁻¹ for glucose uptake.]
value. When transferred to dGlc-free medium, the transport activity resumed in parallel with ATP (Fig. 1). The relationship between ATP level and glucose transport is shown in Figure 2. Similarly, we studied the uptake of fructose and glucose analogs. The data reported in Figure 2 show that there was not much difference between the various sugars when the fractional uptake was plotted versus ATP controlled by dGlc. The fructose (10 mM) was assayed in the presence of unlabeled 3-OMG in order to block its transport by the glucose carrier according to (28). In these conditions, fructose was transported by its specific carrier. Fructose is not a competitor of dGlc for uptake as previously reported (28) and the net efflux of the intracellular dGlc did not depend upon the external fructose concentration (data not shown). This suggests that a direct exchange between the external fructose and the internal dGlc, which might account for the high residual fructose transport activity in the dGlc treatment, is unlikely.

**Effect of Hypoxic Treatment on Adenine Nucleotides and Sugar Transport**

In the course of hypoxic treatment, the ATP level dropped with the pO2, but, unlike the dGlc treatment, the ADP and AMP levels rose, so that the sum of adenine nucleotides remained almost stable (Table I). The relationship between the ATP level, controlled by pO2, and the transport of glucose and dGlc is shown in Figure 2. A 30% decrease in cellular ATP level had no effect on the rate of glucose and dGlc uptake. However, in anoxic root tips, the residual rate of the sugar uptake represented only 25% of its initial value, instead of more than 50% in dGlc treated tips which contained less ATP (Table I). This low rate of sugar uptake under anoxia remained unchanged in the hypoxically acclimated roots having much more ATP.

When ATP-depleted root tips (by dGlc treatment) were placed in anoxic conditions, the residual transport dropped from 50 to 25% of its initial value. These results were the same whatever the prewashing time (5 or 15 min) in dGlc free medium.

**Effect of Fusicox on ATP Level and Sugar Transport**

In the presence of 10 μM FC under normoxic conditions, there was a 50% stimulation of the 3-OMG uptake rate (Table I). Some stimulation was also noted with dGlc treated tips. However, in anoxic conditions, no stimulation of transport by FC could be measured even in non-dGlc treated tips which had twice as much ATP as the treated ones in normoxia (Table I).
The addition of FC induced a 20% decrease in the ATP level of the normoxic or hypoxic root tips (Table II). A similar effect could be seen with root tips which were ATP-depleted by a 30 min pretreatment with dGlc. With longer dGlc pretreatment, the residual ATP level was so low that it was difficult to detect a further decrease caused by FC. In anoxic conditions, there was no FC effect on ATP level.

**Sugar Uptake and H⁺ Symport**

As shown on the traces reported in Figure 3, the addition of exogenous sugars or analogs induced an alkalization of the medium when they were transported. Mannitol, which does not enter the cell, had no effect on the medium pH. Using labeled sugars, we found an apparent stoichiometry of 0.4 mol H⁺·mol⁻¹ for transported 3-OMG and 0.2 for the other sugars assayed.

**Studies on H⁺ Efflux from ATP-Depleted Root Tips**

The functioning of the proton pump was studied in root tips in which the ATP level was controlled by dGlc or pO₂, and was compared with the data obtained for sugar transport. The pH of the incubation medium was recorded in the presence or absence of FC or other additives. The medium was bubbled by gas mixtures containing 5 kPa CO₂.

As shown in Table III, the rate of H⁺ extrusion increased from about 200 pmol H⁺ tip⁻¹·min⁻¹ in the absence of FC to more than 400 pmol tip⁻¹·min⁻¹ in the presence of K⁺ and FC. FC stimulation was tightly dependent upon the K⁺ concentration of the medium as shown in Table III. FC-stimulated acidification could be prevented by 0.4 mM DES or 0.2 mM EB (Fig. 4), whereas vanadate had little effect at the concentration used in our experimental conditions (Fig. 4, b and d).

With dGlc-treated root tips, stimulation by FC decreased with the incubation time in dGlc, and was linearly correlated to the cellular ATP level (Fig. 5). After more than 80 min treatment with dGlc and although ATP level was very low, there was still some FC stimulation of H⁺ extrusion which could be prevented by addition of DES or EB (Fig. 4; Table I). When the ATP level was controlled by pO₂, there was still some FC stimulation at 2.3 kPa O₂ with an ATP level which was half of that in air (Table I). However, once transferred to anoxia the basal acidification fell to zero within minutes and was followed by a slow alkalinization. The addition of FC to unacclimated or acclimated tips did not induce any stimulation of H⁺ extrusion (Fig. 4e; Table IV), despite an ATP concentration, respectively, two or four times higher than in dGlc treated tips (Tables I and IV).

**Effect of Ferricyanide on H⁺ Efflux**

In order to check if a H⁺ extrusion system linked to a redox chain might contribute to the acidification of the medium as described in Elodea densa leaves (10) and in maize root (4, 21), the effect of ferricyanide was examined in the presence or absence of K⁺ or FC in H⁺ efflux experiments on washed root tips. The data reported in Table III show that both K⁺ and FC were needed to promote a large stimulation of H⁺ efflux. The addition of Fe(CN)₆³⁻(BTP salt) slowed down the efflux instead of raising it. Conversely, a large stimulation occurred when K₃Fe(CN)₆ was added after FC; this was not due to the Fe(CN)₆³⁻ but to the large increase in the K⁺ concentration in the medium, which is known to enhance the effect of FC on H⁺ excretion by the membrane ATPase (10, 11, 18). When FC was absent, neither stimulation of H⁺ efflux nor a decrease of cellular ATP was observed in the presence of K₃Fe(CN)₆ for our washed root tips (data not shown).

**Table II. Effect of FC on ATP Level of Root Tips under Different Conditions**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Without FC</th>
<th>10 μM FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1236 ± 50</td>
<td>958 ± 108</td>
</tr>
<tr>
<td>2.3 kPa O₂</td>
<td>568 ± 42</td>
<td>455 ± 47</td>
</tr>
<tr>
<td>N₂ 30 min</td>
<td>303 ± 31</td>
<td>321 ± 34</td>
</tr>
<tr>
<td>dGlc 30 min</td>
<td>348 ± 26</td>
<td>284 ± 18</td>
</tr>
<tr>
<td>dGlc 80 min</td>
<td>166 ± 21</td>
<td>150 ± 30</td>
</tr>
</tbody>
</table>

As Figure 3, the addition of exogenous sugars or analogs induced an alkalization of the medium when they were transported. Mannitol, which does not enter the cell, had no effect on the medium pH. Using labeled sugars, we found an apparent stoichiometry of 0.4 mol H⁺·mol⁻¹ for transported 3-OMG and 0.2 for the other sugars assayed.

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![Figure 3. Sugar-dependent H⁺ uptake in maize root tips. Sixty root tips were placed in 3 mL medium containing 2 mM CaCl₂ and 2 mM KCl. The pH was equilibrated to 5.7 before adding 10 mM sugars (final concentration).](image-url)
Similarly, ferricyanide had no effect in anoxic or hypoxic (2.3 kPa O₂) conditions.

**DISCUSSION**

The data presented are consistent with a H⁺ cotransport of hexoses in excised maize root tips. The low stoichiometry found was probably the result of the H⁺ recycling in the cell surface as demonstrated in (25). As a consequence of this link, any modulation of the H⁺ efflux induced a parallel modification of the rate of hexose uptake.

The two methods used to control the level of cellular ATP in our maize root tips had different consequences on the balance of adenine nucleotides. Under hypoxic conditions, the sum of adenine nucleotides remained nearly constant, and therefore the drop of ATP was balanced by a rise in ADP and AMP. On the other hand, in dGlc treatment, the ADP and AMP levels remained constant as a consequence of the parallel drop of ATP and of the pool of adenine nucleotides. The dGlc is phosphorylated by hexokinases at the expense of ATP to produce nonmetabolisable sugar phosphate as shown in both mammalian cells (12) and maize tissues (23, 28). It has been reported that in ascites tumor cells, dGlc induces a degradation of the adenine nucleotides via the deamination of AMP in inosine monophosphate and subsequent dephosphorylation to produce inosine (13). Similar reactions might have occurred in our root tips.

Unlike anoxic root tips in which both sugar transport and H⁺ efflux were abolished, there was still a high residual rate of sugar uptake in tips treated by dGlc and the H⁺ efflux persisted although the level of ATP was about half that in anoxic roots (Table I). This difference between both treatments might be attributed to the involvement of some O₂ and NAD(P)H dependent redox chain coupled to proton secretion and activated by ferricyanide, as described by several authors (4, 10, 16, 21). However, the lack of stimulation of H⁺ extrusion by ferricyanide either in normoxic or in hypoxic conditions makes its contribution to membrane polarization unlikely.

According to (14) and (26), the apparent Kₘ of the electrogenic pump for ATP, measured in intact cells, ranges from 10 to 100 μM and therefore the residual level of ATP in ATP-depleted tissues (about 160 and 300 μM in root tips treated by dGlc or in anoxia, respectively, assuming 1 μL of sap per 2.2 mg tip) could be high enough to allow a reasonable membrane hyperpolarization in the absence of any other inhibitors.

The idea that H⁺ extrusion in dGlc-treated tips depends on H⁺-ATPase activity is supported by several pieces of evidence:

(a) H⁺ efflux was stimulated by FC in the presence of K⁺;
(b) this stimulated efflux was blocked by ATPase inhibitors;
(c) the FC stimulation of both sugar transport and H⁺ exit was tightly linked to the cellular ATP level.

The poor effect of vanadate on H⁺ exit may be attributed to its poor penetration into the intact tissues. Brummell *et al.* (5) have reported that a higher concentration (1 mM) and a longer incubation (more than 2 h) are necessary, *in vivo*, to block the acidification of the medium, and that the efficiency of *in vivo* vanadate inhibition of H⁺-pump depends upon the plant species.

In anoxic treatments, and despite twice as much ATP as in dGlc tips, there was no FC stimulation on either sugar transport or H⁺ efflux. Root tips excised from hypoxically adapted seedlings according to Saglio, *et al.* (24) did not show any improvement in sugar uptake and H⁺ extrusion under anoxic conditions (Table IV), despite their higher cellular ATP con-

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**Table III. Effects of Fe(CN)₆⁻³ or K⁺ on Medium Acidification**

H⁺ exit was measured as described in "Materials and Methods" with 80 root tips in 3 ml medium containing 0.5 mM Mes and CaSO₄, bubbled by a gas mixture composed of 70, 25, and 5 kPa of N₂, O₂, and CO₂, respectively. The starting pH was adjusted to 6.0 by BTP. The reagents were added successively, at a final concentration of 10 μM for FC and 1 mM for the others. H⁺ exit is expressed in pmol tip⁻¹ min⁻¹.

<table>
<thead>
<tr>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
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</thead>
<tbody>
<tr>
<td>Successful addition</td>
<td>Net H⁺ exit</td>
<td>Successful addition</td>
</tr>
<tr>
<td>0</td>
<td>251</td>
<td>0</td>
</tr>
<tr>
<td>FC</td>
<td>279</td>
<td>Fe(CN)₆⁻³</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>485</td>
<td>FC</td>
</tr>
<tr>
<td>K₃Fe(CN)₆</td>
<td>619</td>
<td>K₂SO₄</td>
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</table>

**Figure 4.** Effects of inhibitors on FC-dependent H⁺ exit. Root pretreatment and incubation medium were the same as in Table I except that the medium contained 9 mm KCl. For the vanadate assay (b, d), the root tips were pretreated for 40 min with 0.3 mM vanadate prepared as in (17), and the subsequent H⁺ efflux measurement was performed in the presence of the same concentration of vanadate. EB was prepared in water as Na⁺ salt (pH 6.0). FC and DES were dissolved in 95% ethanol. The added ethanol in the medium did not exceed 0.7%. The starting pH was 5.8.
tent (about 565 μM). In both cases, the inhibition of H⁺ efflux occurred within minutes after the beginning of the anoxic treatment, as observed for hypoxic membrane depolarization of wheat roots (7).

As demonstrated by Blatt and Clint (3 and references therein), membrane potential is a function of both H⁺-pump activity and membrane conductance, and membrane hyperpolarization induced by FC can be ascribed to a reduction of the conductance without the necessity for postulating a large stimulation on H⁺ pumping. Accordingly, the remaining transport and H⁺ extrusion activities in dGlc-treated root tips could be alternatively interpreted as a result of a decrease in membrane conductance. However, unless another energy-coupling mechanism is involved in the conductance modulation, the question arises how such a high residual transport activity, stimulated by FC, at very low cellular ATP levels (Table I; Fig. 2), can be maintained? Another question is: why FC had no effect at all under anoxic conditions which are known to modify conductance by enhancing ion leaks?

It is worth noting that anoxic cells did not use their residual ATP for the regeneration of membrane potential. Such inhibition of membrane potential has been attributed to ADP in perfused Chara (26), and in vivo studies on membrane fractions of Beta vulgaris have shown a competitive inhibition of plasma membrane ATPase by ADP but not by AMP (2). The relationship between adenine nucleotides and membrane potential has been studied in detail in Nitellopsis obtusa using a continuous perfusion method (15). It is shown that both ADP and AMP are competitive inhibitors of the electrogenic pump with a $K_i$ of about 0.4 mM. In our anoxic roots, the ADP increased from 0.19 to 0.45 mM and the AMP from 0.03 to 0.55 mM whereas in dGlc treatment their concentrations hardly changed even when the ATP dropped to 15% of its initial level. These results may account for the inhibition of H⁺ efflux and sugar transport under anoxic conditions.

An alternative explanation is that Pi which rose in anoxic cells could be involved in the inhibition of the activity of H⁺-ATPase as reported in (2, 8). In the presence of dGlc, the trapped Pi might not exert its inhibitory effect.

In conclusion, our results demonstrate that both the membrane hyperpolarization and the second transport activity can persist at very low cellular energy status and the primary factor for maintenance of these activities in maize root tips could be ascribed to the activity of plasma ATPase, which could be modulated by ADP and/or AMP. However, the action of Pi or other unknown factors produced under anoxic conditions cannot be excluded.

ACKNOWLEDGMENT

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LITERATURE CITED