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Pyrophosphate and H^+-pyrophosphatase maintain the vacuolar proton gradient in metabolic inhibitor-treated *Acer pseudoplatanus* cells

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Abstract

The effect of metabolic inhibitors (KCN and 2-deoxy-D-glucose) on the vacuolar proton gradient, monitored by acridine orange, was assayed in *Acer pseudoplatanus* cells. Potassium cyanide plus 2-deoxy-D-glucose slightly lowered this gradient, while cellular ATP level was strongly decreased and inorganic pyrophosphate (PP_i) content was halved. Two phosphatase inhibitors (imidodiphosphate and KF) restored the PP_i level in KCN-treated cells, but decreased the vacuolar proton gradient by inhibiting H^+-PP_iase. These results, hence, suggest that tonoplast H^+-PP_iase is especially responsible for the maintenance of vacuolar ΔpH and that this enzyme is the major scavenger of cytoplasmic PP_i in cells treated with metabolic inhibitors.

Keywords: Pyrophosphatase, H^+-; Metabolic inhibitor; Proton gradient; Pyrophosphate; Vacuole; (*A. pseudoplatanus* cells)

1. Introduction

Unlike animal cells, plant cells survive after long periods of hypoxia, because they carry out only a transient lactic fermentation which provides the signal to trigger the ethanolic fermentation [1]. As known, the latter does not result in a severe acidosis [2]. Many plant cells indeed tolerate short periods of anaerobiosis because of their ability to maintain a constant vacuolar pH [3,4]. In non-tolerant plants, cell death is caused by a leakage of acids from vacuoles which determines a cytoplasmic acidosis [5].

The vacuolar function is linked to the activities of H^+-ATPase (EC 3.6.1.3) and H^+-PP_iase (EC 3.6.1.1) which build up an electrochemical gradient across the tonoplast, utilizing ATP or pyrophosphate (PP_i) as substrates [6]. Since the cytoplasmic PP_i level, unlike that of ATP, does not change when tissues are subjected to anoxia or respiratory poisoning [7,8], it has been suggested that this energy source is utilized by H^+-PP_iase to maintain vacuolar compartmentation during transient metabolic perturbations [9]. In addition, this enzyme, endowed with a high affinity for PP_i, can also be active at very low substrate concentrations [6] and appears to be inducible by either anoxia or chilling [10].

In the present work we studied the effect of 2-deoxy-D-glucose (a glycolysis inhibitor) and KCN (a respiration inhibitor) on vacuolar ΔpH and cellular ATP or PP_i levels of *Acer pseudoplatanus* cells.

2. Materials and methods

Cell culture. Submerged cultures of *A. pseudoplatanus* L. cells were grown, as described in Ref. [11], for 6 days, at 25° C, in a rotatory bath (120 rev/min). Cells were collected by filtration through a Buchner filter No. 3 under vacuum and then resuspended in 25 mM Tris-Mes (pH 7.5), 0.7 M mannitol, 150 mM KBr and 4 mM $MgSO_4$ to a final concentration of 25 mg FW/ml.

Vacuole preparation. Vacuoles were isolated from protoplasts, obtained as described previously [12], following the method of Pugin et al. [13]. Vacuoles were resuspended in 25 mM Tris-Mes (pH 7.5), 0.7 M mannitol, 150

Abbreviations: AO, acridine orange; BAF, bafilomycin A_1; FC, fusicoccin; FCCP, carbonyl cyanide *p*-trifluorometoxyphenylhydrazone; FW, fresh weight; IDP, imidodiphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PP_i, inorganic pyrophosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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mM KBr and 4 mM $MgSO_4$ to a final concentration of approx. $2 \cdot 10^5$ vacuoles/ml.

Acridine orange assay. The uptake of AO was continuously followed, at room temperature, as a decrease of the absorbance difference (495–540 nm) by a double-beam/double-wavelength Perkin-Elmer spectrophotometer, model 356. The medium was: 25 mM Tris-Mes (pH 7.5), 0.7 M mannitol, 150 mM KBr, 4 mM $MgSO_4$ and 50 mg (FW) of cells or 10^5 vacuoles in a final volume of 2 ml. The uptake was started by the addition of 5 μM AO.

The vacuolar ΔpH (proton gradient established between vacuole and external medium) was determined as a NH_4^+-induced release of AO from pre-loaded cells. The latter were incubated for 15 min in the above medium containing 5 μM AO. At the end of the incubation, NH_4^+-induced release of dye (30 mM $(NH_4)_2SO_4$ addition) was determined as increase of AO absorbance.

Determination of ATP and PP_i. Cells were collected by a Buchner filter No. 3, rapidly frozen under liquid N_2 and then ground in a mortar with 2 ml/g FW of 0.45 M perchloric acid without incubation [14]. The precipitate was removed by centrifugation and the supernatant neutralized with triethanolamine/KOH. The potassium perchlorate was removed by cold precipitation, followed by centrifugation. The final supernatant was collected for ATP or PP_i determinations.

Adenosine 5′-triphosphate was determined using the hexokinase and glucose-6-phosphate dehydrogenase method, as described by Lamprecht and Trautschold [15], whereas PP_i level was measured following the method described by Smith and Black [14]. The concentration of ATP or PP_i was obtained using standard calibration curves.

Chemicals. All chemicals were purchased from Sigma Co. St Louis, MO, USA. Bafilomycin A_1 (BAF) was a gift of Dr. H.P. Fiedler, University of Tübingen, Germany. Fusicoccin (FC) was a gift of Prof. E. Marrè, University of Milan, Italy. Potassium cyanide was dissolved to give a 1 M stock solution which was brought to pH 7.5 with Mes.

Data presentation. Results of Fig. 1 are representative of a typical experiment. Other data are means of three replicates $\pm$S.D.

3. Results

3.1. Acridine orange uptake by A. pseudoplatanus cells

Acridine orange is currently used to monitor proton gradients in isolated membrane vesicles from plants and animals [16,17], and in animal cells [18].

The addition of AO to a *A. pseudoplatanus* cell suspension caused a time-independent increase of absorbance followed by a progressive decrease (Fig. 1, trace A). The subsequent addition of NH_4^+ determined a new increase of dye absorbance. Nigericin or FCCP, added when the AO absorbance decrease had reached a steady

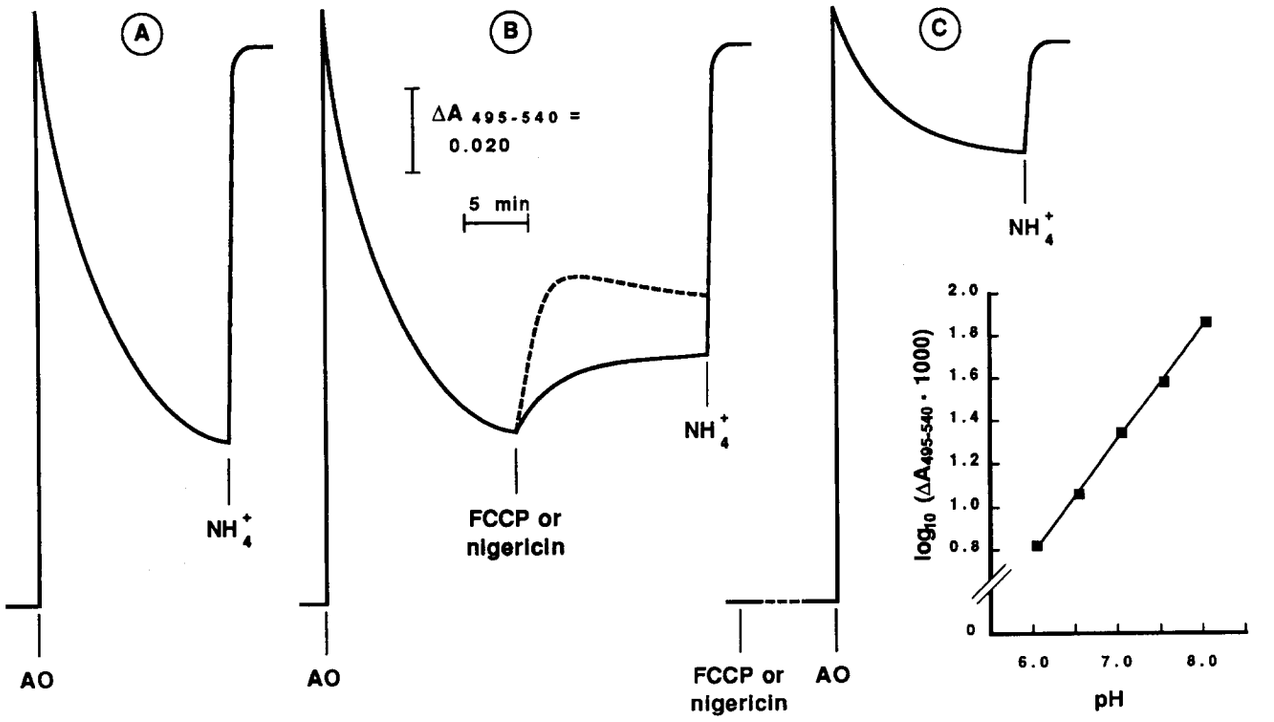

Fig. 1. Acridine orange uptake by *A. pseudoplatanus* cells. Additions: 5 μM AO; 30 mM $(NH_4)_2SO_4$; 5 μM FCCP (solid line); 25 μM nigericin (dashed line). Inset represents NH_4^+-induced AO release versus external pH variations.

state, only partially reversed this decrease (Trace B). On the contrary, a consistent reduction of the decrease of AO absorbance was observed when cells were pre-incubated for 15 min with these ionophores (Trace C). Ammonium-induced AO release (log scale) was linearly correlated to the difference of pH, established between the external medium and the interior of the vacuole, in the 6.0–8.0 pH range of the incubation mixture (Fig. 1, inset). In agreement with these results, the observation of AO-treated cells by fluorescence microscopy showed a red fluorescence inside the vacuole, while the colour shifted to green after NH_4^+ addition (Fig. 2).

These observations suggest that there was an accumulation of AO in the acidic compartment of the cells (vacuole) and that the subsequent alkalinization of the vacuole caused the release of the dye. Therefore, AO appears to be a suitable probe for proton gradient measurements not only in reconstituted plant membranes [16], but also in plant cells.

3.2. Effect of H^+-ATPase and H^+-PP_iase inhibitors and FC on H^+ extrusion and vacuolar ΔpH (NH_4^+-induced release of AO) in A. pseudoplatanus cells

As the low vacuolar pH is maintained by the activity of H^+-ATPase and H^+-PP_iase [19], inhibitors of these pumps should decrease AO accumulation. Bafilomycin A_1 (a vacuolar H^+-ATPase inhibitor) [20], IDP (an H^+-PP_iase competitive inhibitor) [21] and KF (an H^+-PP_iase inhibitor) [22] were assayed. Fluoride also inhibits H^+-ATPase, but only at very high concentrations [23].

Indeed, after 3 h incubation, BAF and IDP lowered NH_4^+-induced release of AO, without affecting the acidification of the external medium. Bafilomycin A_1 plus IDP caused a more pronounced inhibitory effect (Table I). Their specificity was underlined by their lack of effect on H^+ extrusion and was further supported by the additive effect of BAF and IDP, indicating that AO accumulation depended on the activity of both H^+ pumps.

Table 1
Effect of tonoplast H^+-ATPase and H^+-PP_iase inhibitors and FC on proton extrusion and vacuolar ΔpH in *A. pseudoplatanus* cells after 3 h incubation.

Additions	H^+ extrusion (ext. pH)	ΔpH [a] ($\Delta A_{495-540}$)
Control (0 h)	7.50	0.094 ± 0.010
Control (3 h)	7.34 ± 0.03	0.082 ± 0.008 [b]
20 μM BAF	7.36 ± 0.05	0.054 ± 0.009 [b]
10 mM IDP	7.38 ± 0.02	0.038 ± 0.012 [b]
BAF + IDP	7.25 ± 0.04	0.018 ± 0.006 [b]
20 μM FC	7.23 ± 0.03	0.030 ± 0.008 [b]

[a] ΔpH was measured as NH_4^+-induced release of AO.
[b] After 3 h incubation, the external pH of the samples was restored to the initial value (pH 7.5) by addition of Tris. Then, 5 μM AO was added to load cells with the dye. After 15 min, 30 mM $(NH_4)_2SO_4$ was supplied and NH_4^+-induced release of AO determined as $\Delta A_{495-540}$.

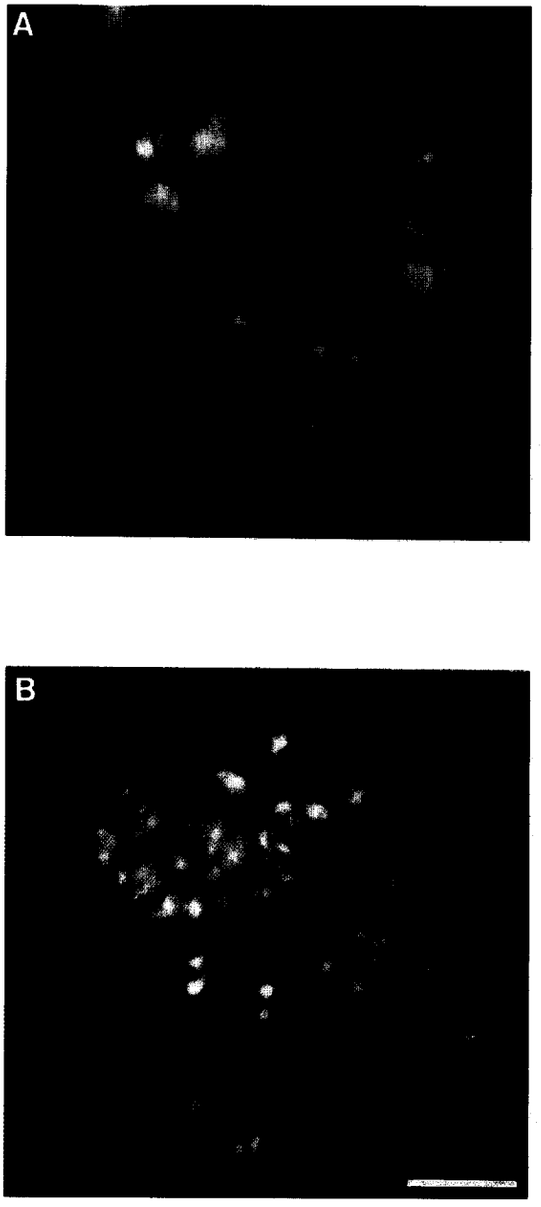

Fig. 2. Fluorescence microscopy of AO-treated *A. pseudoplatanus* cells before (panel A) or after (panel B) NH_4^+ addition. ×150; bar = 100 μm.

Table 1 also shows that FC, stimulating H^+ extrusion [24], significantly acidified the external medium. This effect was accompanied by a strong decrease in vacuolar ΔpH, in agreement with previous reports [25–27].

3.3. Effect of metabolic inhibitors on H^+ extrusion and vacuolar ΔpH (NH_4^+-induced release of AO) in A. pseudoplatanus cells

The activity of vacuolar proton pumps, both ATP and PP_i-driven, depends on the continuous supply of these substrates. For this reason, the effect of metabolic inhibitors (2-deoxy-D-glucose and KCN) on H^+ extrusion

Table 2
Effect of metabolic inhibitors on proton extrusion and vacuolar ΔpH in *A. pseudoplatanus* cells after 24 h incubation

Additions	H^+ extrusion (ext. pH)	ΔpH [a] ($\Delta A_{495-540}$)
Control (0 h)	7.50	0.088 ± 0.008
Control (24 h)	7.22 ± 0.09	0.077 ± 0.011 [b]
10 mM KCN	7.47 ± 0.07	0.063 ± 0.005 [b]
5 mM 2-deoxy-D-glucose	7.25 ± 0.07	0.072 ± 0.007 [b]
KCN + 2-deoxy-D-glucose	7.52 ± 0.04	0.061 ± 0.006 [b]

[a] ΔpH was measured as NH_4^+-induced release of AO.
[b] After 24 h incubation, the external pH of the samples was restored to the initial value (pH 7.5) by addition of Tris. Then, 5 μM AO was added to load cells with the dye. After 15 min, 30 mM $(NH_4)_2SO_4$ was supplied and NH_4^+-induced release of AO determined as $\Delta A_{495-540}$.

Table 3
Effect of ATP and PP_i on ΔpH of isolated *A. pseudoplatanus* vacuoles after 3 h incubation

Additions	ΔpH [a] ($\Delta A_{495-540}$)
Control (0 h)	0.102 ± 0.015
Control (3 h)	0.053 ± 0.009 [b]
1 mM ATP	0.090 ± 0.013 [b]
100 μM PP_i	0.068 ± 0.011 [b]
1 mM ATP + 100 μM PP_i	0.094 ± 0.009 [b]
1 mM ATP + 10 μM BAF	0.042 ± 0.007 [b]
100 μM PP_i + 500 μM IDP	0.038 ± 0.005 [b]

[a] ΔpH was evaluated as NH_4^+-induced release of AO.
[b] After 3 h incubation, vacuoles were loaded with 5 μM AO for 15 min and NH_4^+-induced release of AO was determined by monitoring $\Delta A_{495-540}$ induced by the addition of 30 mM $(NH_4)_2SO_4$.

and vacuolar ΔpH was studied (Table 2), to simulate conditions of anoxia or glucose starvation.

Incubation of control cells for 24 h determined an acidification of the incubation medium which was associated with a low decrease in vacuolar ΔpH. Potassium cyanide inhibited H^+ extrusion, while only slightly lowering the vacuolar ΔpH. In contrast, 2-deoxy-D-glucose caused negligible changes in both H^+ extrusion and vacuolar ΔpH. This indicates that ATP, produced by glycolysis, was not essential in maintaining the vacuolar proton gradient. In agreement with this, KCN plus 2-deoxy-D-glucose showed an effect comparable to that caused by KCN alone.

3.4. Role of PP_i in maintaining vacuolar ΔpH (NH_4^+-induced release of AO) in A. pseudoplatanus cells treated with metabolic inhibitors

The low impairment of vacuolar ΔpH, caused by KCN, raises the question as to whether this could be maintained by PP_i and/or ATP, substrates of vacuolar H^+-PP_iase and H^+-ATPase, respectively. In anaerobiosis, the PP_i level is maintained constant [7], while ATP may be produced by glycolytic reactions [1].

Table 3 shows that the ΔpH of isolated vacuoles was approx. 50% decreased after 3 h of incubation. The additions of ATP and PP_i, separately or together, partially (PP_i) or almost completely (ATP) restored this ΔpH. The presence of ATP or PP_i, hence, appears to be crucial for maintaining the proton gradient across the tonoplast. In agreement with this, BAF or IDP inhibited, respectively, the ATP- or PP_i-dependent restoration of ΔpH. These results confirm, in agreement to what was found by others [28], that the H^+-PP_iase seems to have a minor role, when compared to the H^+-ATPase, in maintaining the vacuolar ΔpH.

The incubation of control cells for 24 h induced a negligible decrease of ATP and an increase of PP_i level. Potassium cyanide alone or plus 2-deoxy-D-glucose caused, respectively, an approx. 95% and 38% decrease in cellular concentrations of ATP and PP_i (Table 4).

The large drop in the ATP level, caused by KCN, as a consequence of respiratory inhibition, explains the lack of acidification of the medium in KCN-treated cells (Table 2); the latter depends on the diminished availability of ATP which limits the activity of the plasmalemma H^+-ATPase. Also, the decreased level of PP_i may be linked to the drop in ATP content, since several cell biosynthetic reactions, yielding PP_i, depend on a continuous supply of nucleotide triphosphates [29].

The decrease of PP_i level in KCN-treated cells is somewhat puzzling. To clarify this point, the effect of two PP_iase inhibitors (KF and IDP) on PP_i level and vacuolar ΔpH in metabolic inhibitor-treated cells was assayed (Table 5). Metabolic inhibitors, as above reported, lowered the PP_i level, but did not greatly decrease vacuolar ΔpH. Fluoride and IDP, per se, strongly increased the PP_i level of cells [30,31] while lowering the proton gradient. In the KCN plus 2-deoxy-D-glucose-treated cells, the level of PP_i was partially (KF) or almost completely (IDP) restored by PP_iase inhibitors, whereas ΔpH was more strongly lowered. Therefore, the inhibition of H^+-PP_iase was crucial in

Table 4
Effect of metabolic inhibitors on ATP and PP_i level of *A. pseudoplatanus* cells after 24 h incubation

Additions	ATP level (nmol g^{-1} FW)	PP_i level (nmol g^{-1} FW)
Control (0 h)	128 ± 18	13 ± 3
Control (24 h)	101 ± 13	21 ± 5
10 mM KCN	7 ± 2	11 ± 2
10 mM KCN + 5 mM 2-deoxy-D-glucose	5 ± 1	8 ± 3

Table 5
Effect of KF and IDP on vacuolar ΔpH and PP_i level in metabolic inhibitor-treated *A. pseudoplatanus* cells after 24 h incubation

Additions	ΔpH [a] ($\Delta A_{495-540}$)	PP_i level (nmol/g^{-1} FW)
Control (0 h)	0.090 ± 0.012	11 ± 2
Control (24 h)	0.080 ± 0.010 [b]	20 ± 4
10 mM KCN + 5 mM 2-deoxy-D-glucose	0.066 ± 0.006 [b]	7 ± 2
5 mM KF	0.064 ± 0.005 [b]	52 ± 9
5 mM IDP	0.040 ± 0.005 [b]	87 ± 12
KCN + 2-deoxy-D-glucose + KF	0.018 ± 0.003 [b]	23 ± 5
KCN + 2-deoxy-D-glucose + IDP	0.020 ± 0.002 [b]	81 ± 10

[a] ΔpH was measured as NH_4^+-induced release of AO.
[b] See Table 2.

preventing PP_i consumption and causing a parallel decrease in vacuolar ΔpH.

4. Discussion

Acridine orange has been used to monitor proton gradients in parietal cells [33], secretory granules of β-cells [33] and rat thymocytes [18]. The dye is also taken up by *A. pseudoplatanus* cells. The accumulated AO is completely released by NH_4^+, partially by nigericin and, to a minor extent, by FCCP. Dye uptake is decreased by specific inhibitors of vacuolar H^+-ATPase (BAF) or H^+-PP_iase (IDP and KF). These results indicate that AO is accumulated in the acidic compartment of the cells in response to the proton gradient established between the vacuole and the external medium (vacuolar ΔpH). The slight effect of FCCP should depend on the fact that the protonophore (uncoupler) collapses the protonmotive force, but not the ΔpH which is still maintained by the presence of organic acids in the vacuole [34].

Although H^+-ATPase and H^+-PPase are present in the tonoplast, the relative role of these pumps is still controversial. On the basis of considerations of equilibrium thermodynamics [6], or experimental evidence [35], it was inferred that the former, which has a H^+/ATP stoichiometry of 2, would act physiologically as a pump, while the electrochemical proton gradient it generates would be used by the latter (H^+/PP_i stoichiometry ratio of 1), to synthesize PP_i and, hence, stabilize cytoplasmic PP_i level. The results on vacuoles show that ATP is more efficient than PP_i in preventing the decrease of ΔpH. This suggests that, in agreement with the above statement, the H^+-ATPase has a predominant role in maintaining vacuolar ΔpH. In addition, a recent paper [28] showed that the light-stimulated proton pumping does not require the activity of tonoplast H^+-PP_iase. Apparently, the proton pumping can be based solely on the activity of the tonoplast ATPase. Therefore, the H^+-PP_iase should be important under conditions of limited ATP supply, exerting only an ancillary role during anaerobiosis [36].

Metabolic inhibitors (KCN and 2-deoxy-D-glucose), used separately or together, have only a slight inhibitory effect on vacuolar ΔpH. On the contrary, KCN or KCN plus 2-deoxy-D-glucose strongly decrease cellular ATP level, while halving the PP_i content. The vacuolar ΔpH is hence maintained even in cells depleted of ATP by metabolic inhibitors, according to what was found in cells grown in anaerobiosis [3,4]. While the drop of ATP in metabolic inhibitor-treated cells is expected, the decrease of PP_i is, at least in part, surprising because it does not occur in tissues subjected to anoxia [7]. As PP_i is synthesized through several biosynthetic reactions requiring ATP [28], its decreased level may depend on the low availability of the latter. On the other hand, PP_i can be consumed to sustain the vacuolar H^+-PP_iase activity which, as suggested [9], would maintain the proton gradient in anoxic cells. The latter hypothesis is supported by the experiment with inhibitors of phosphatases (KF and IDP).

In agreement with the results obtained by others [30,31], KF and IDP increase the PP_i content in untreated cells. In addition, these inhibitors restore the PP_i level in cells treated with metabolic inhibitors, while decreasing the vacuolar ΔpH by inhibition of the H^+-PP_iase. It is therefore concluded that the tonoplast H^+-PP_iase is especially responsible for the maintenance of vacuolar ΔpH and that this enzyme is the major scavenger of PP_i in the metabolic inhibitor-treated cells. The pronounced sensitivity of the H^+-PP_iase to free Mg^{2+} [36], which increases in sodium azide-treated cells [37], makes this enzyme particularly useful to maintain vacuolar ΔpH under conditions of limited ATP supply (e.g., anaerobiosis). This notion is supported by the observation that H^+-PP_iase is induced by anoxia or chilling and, consequently, plays a key role in maintaining the vacuolar proton gradient and in limiting cytoplasmic acidosis [10].

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