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ATP synthesis and pyrophosphate-driven proton transport in tonoplast-enriched vesicles isolated from *Catharanthus roseus*

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In the presence of PP_i as an energy source, the tonoplast-bound inorganic pyrophosphatase from *Catharanthus roseus* cells is able to create a proton-gradient which can drive the synthesis of ATP from ADP and P_i. ATP synthesis is linked to the pH-gradient dissipation as monitored by the recovery of the fluorescence intensity of quinacrine and by the amount of synthesized ATP measured by the bioluminescent luciferin/luciferase assay. Proton gradient and ATP synthesis were suppressed by the protonic ionophore gramicidin D.

Tonoplast vesicle; Pyrophosphatase; ATP synthesis; (*Catharanthus roseus*)

1. INTRODUCTION

Inorganic pyrophosphatases have been found in higher plant cells and were described as energy donors, that is plant cells can utilize PP_i instead of ATP to drive electrogenic proton transport across the tonoplast via an H^+-pyrophosphatase [1]. However, up to now, no clear evidence was given for energy-linked reactions or for secondary transports of solutes into the acidified vesicles. Here, we demonstrate that the PPase, functioning as a proton-pump, can drive the synthesis of ATP from ADP and inorganic phosphate in tonoplast-enriched vesicles prepared from *Catharanthus roseus* cultured cells.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BTP, bis-tris-propane; Ap^5A, P_1,P_5-di(adenosine-5′) pentaphosphate; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PPase, inorganic pyrophosphatase

2. MATERIALS AND METHODS

ATP, ADP, AMP and Ap^5A were purchased from Sigma. Luciferin, luciferase were from Boehringer. All other chemicals were purchased from Merck.

2.1. *Plant material*

The cell line C20 of *Catharanthus roseus* (L.) G. Don were grown at 28°C in B5 medium [2] containing 1 μM 2,4-dichlorophenoxyacetic acid and 60 nM kinetin. All experiments were performed with 5- or 6-day-old suspensions.

2.2. *Preparation of the vesicles*

The preparation and characterization of tonoplast-enriched vesicles have already been described [3]. Briefly, the homogenization medium consisted of 50 mM Hepes-BTP, 250 mM sorbitol, 2.5 mM DTT, 3 mM EGTA, 0.1% (w/v) BSA (fraction V, essentially fatty acid free) at pH 7.5. The suspension medium consisted of 25 mM Hepes-BTP, 250 mM sorbitol, 2.5 mM DTT at pH 7.5. It was made 5 mM Hepes-BTP for fluorescence quenching kinetics. All operations were carried out at 0–4°C. The cells (50 or 100 g fresh wt) were homogenized with a 60 ml potter homogenizer (Braun Melsungen) using a medium to cell ratio of 1:1.5 ml/g. The bree was filtered through a Büchner and centrifuged for 10 min at $1000 \times g_{av}$. To remove mitochondrial fraction, the supernatant was centrifuged twice at $12000 \times g_{av}$ for 15 min. Membranes in the resulting supernatant were then pelleted by centrifugation at $100000 \times g_{av}$ for 30 min (Sorvall, T865 rotor). The microsomal pellet was resuspended in 6–8 ml suspension medium to a protein concentration of 4–5 mg/ml, using a

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manual potter homogenizer (no. C 14477, Thomas Co., Philadelphia). Aliquots of 6–8 ml were layered on top of two step gradients of 20 ml of 10% (w/v) dextran T70 and 10 ml of 50% (w/v) saccharose, both in suspension buffer. After centrifugation in a swing-out rotor at 50000 × g_{av} for 2 h (Beckman, SW 25.2), the membranes at the buffer-dextran interface, F1 fraction, were collected with a Pasteur pipette. Then, this fraction was diluted ten times in suspension medium and centrifuged at 100000 × g_{av} for 30 min. The resulting pellet, resuspended in 2 ml suspension medium, was used for further experiments.

2.3. *PPase activity*

The quinacrine fluorescence quenching kinetics were performed at 20°C on an Eppendorf fluorimeter, equipped with 405 and 530 nm interferential filters for excitation and emission, respectively. The collection of data was performed by the collection unit of the 'GS-Digiview' program, through an analog-digital converter-timer board for Apple II provided by Digimetrie (Perpignan, France). Calculations and graphs were obtained with the processing unit of the same program [4]. Tonoplast-enriched vesicles were added to 0.6 ml of 5 mM Hepes-BTP buffer containing 2.5 mM DTT, 10 mM $MgCl_2$, 50 mM KCl and 0.25 M sorbitol. After 5 min incubation, the reaction was initiated by addition of 5–10 μl ATP or PP_i stock solutions, neutralized by solid bis-tris-propane.

2.4. *ATP synthesis*

The amount of synthesized ATP was measured with the luciferin/luciferase technique in a luminometer that we have previously described [5]. The reaction was triggered by adding 10 μl of ADP and PP_i stock solutions in a plastic cuvette containing 1.1 ml of 50 mM Hepes-BTP buffer, 2 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, 0.15 mM AMP, 0.3 mM luciferin and 0.85 μg luciferase at pH 7.75. The light output was calibrated by addition of known amounts of ATP.

2.5. *Protein assay*

Proteins were determined using the method of Bradford [6] modified according to [7] with BSA as a standard.

3. RESULTS AND DISCUSSION

3.1. *PP_i-dependent proton-pump*

With $MgCl_2$ and KCl fixed at 10 and 50 mM, respectively, the apparent K_m for PP_i-dependent H^+-pumping was 22 μM at pH 7.95 (fig.1), similar to values reported for red beet [8] and for oat root [9] vesicles.

Table 1 summarizes the effects of different compounds tested on the H^+-pumping pyrophosphatase. Oligomycin and sodium orthovanadate had no effect on the proton pump while KNO_3 activated the proton uptake. The results published in the literature on the effects of nitrate are contradictory. In agreement with [10,11], we found that PP_i-dependent proton pumping by tonoplast membranes was activated by nitrate (fig.2) while inhibitory [8] or no effect [12] were also described. Surprisingly, AP^5A, which is known as a potent in-

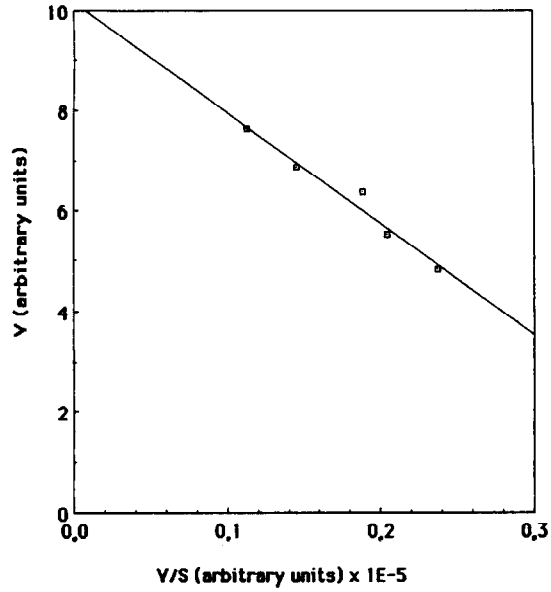

Fig.1. Effect of PP_i on H^+-pumping into tonoplast vesicles. The Woolf plot gave an apparent K_m for PP_i of 22 μM. Experimental conditions: suspension medium, 0.6 ml, contained 5 mM Hepes-BTP at pH 7.95, 0.25 M sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM DTT, 8 μM quinacrine, 44 μg protein. Reactions were triggered by PP_i.

Table 1

Effect of different compounds on the H^+-pumping pyrophosphatase

Compounds	Concentration	Fluorescence quenching (% control)
Sodium molybdate	1 mM	86.5
Ap^5A	1 mM	22
ADP	0.165 mM	73
AMP	0.24 mM	76
P_i	0.76 mM	54
Potassium nitrate	75 mM	142
Oligomycin	10 μg/ml	112
Sodium orthovanadate	0.1 mM	101

PP_i-dependent H^+-pumping was monitored as initial rate of quenching of quinacrine fluorescence. The suspension medium, 0.6 ml, contained 5 mM Hepes-BTP at pH 7.5, 0.25 M sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM DTT, 8 μM quinacrine, 109 μg protein. Reactions were triggered by 0.76 mM PP_i

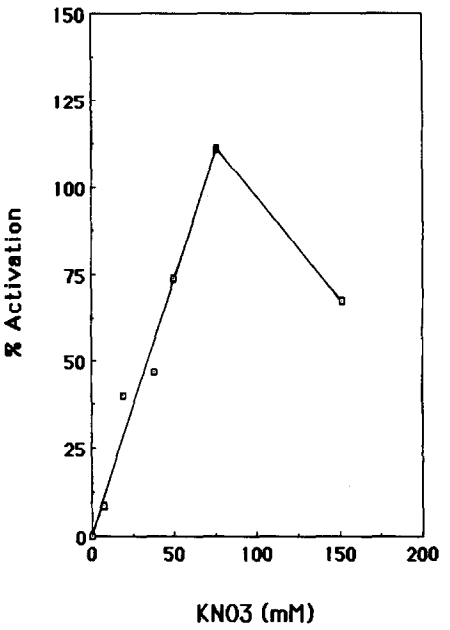

Fig.2. Effect of KNO_3 on PP_i-dependent proton pump in tonoplast vesicles. Experimental conditions: suspension medium, 0.6 ml, contained 5 mM Hepes-BTP at pH 7.95, 0.25 M sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM DTT, 8 μM quinacrine, 55 μg protein. Reactions were triggered by PP_i at 0.76 mM, final concentration.

hibitor of adenylate kinase [13,14] strongly inhibited H^+-proton pumping. Inorganic phosphate inhibited the formation of the pH gradient at a 46% rate, whereas AMP and ADP had little effect. Moreover, ADP did not induce proton uptake. Thus, in the case of tonoplast vesicles from *Catharanthus roseus*, the H^+-PPase is not associated with a H^+-ADPase activity as found for pea stem tonoplast vesicles [12].

3.2. *PP_i-driven ATP formation*

Two different methods were used to measure ATP synthesis: kinetics of quinacrine fluorescence and the bioluminescence assay.

A coupled mechanism between pyrophosphate-driven proton transport and ATP synthesis implies that the fluorescence quenching of quinacrine must be reversed in the presence of ADP and P_i. Indeed, the pH gradient induced by PP_i hydrolysis was dissipated during ATP synthesis (fig.3). When the pH gradient reached its maximum value, the addition of ADP restored the quinacrine fluorescence. AMP had no effect.

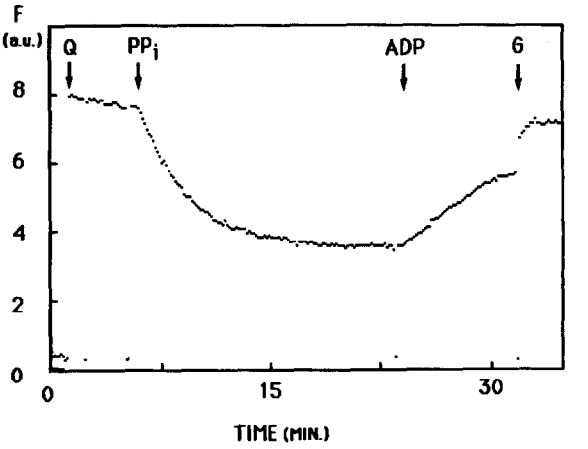

Fig.3. Effect of ADP on H^+-pumping pyrophosphatase: fluorescence intensity (F) in arbitrary units vs time. Experimental conditions: suspension medium, 0.6 ml, contained 5 mM Hepes-BTP at pH 7.95, 0.25 M sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM DTT, 100 μg protein. The arrows correspond to addition of 8 μM quinacrine (Q); 0.76 mM PP_i; 0.165 mM ADP; 5 μg/ml gramicidin D (G), final concentrations.

The time course of ATP formation in tonoplast-enriched vesicles, observed by the bioluminescence assay, is illustrated in fig.4. Curve 1 represents the amount of synthesized ATP when ADP was added after 15 min incubation of the vesicles with PP_i, i.e. when the proton gradient had reached its maximum value, while curve 2 corresponds to ATP synthesis without preliminary incubation. In the latter case, the level of ATP formation was inferior because of the competition between PP_i-driven ATP synthesis and ATP consumption by the luciferin/luciferase reaction. Without tonoplast vesicles, a low bioluminescent signal was observed upon addition of ADP. It corresponded to ATP contamination of the ADP in the sample (2–3%). With tonoplast vesicles and without PP_i, we observed the same low signal; thus, under our experimental conditions, we could not detect the tonoplast-bound ATPase.

The antibiotic protonophore, gramicidin D at 5 μg/ml, completely collapsed the proton gradient and suppressed the ATP synthesis proving that the formation of ATP is driven by the proton gradient created by the PPase. In the bacterium, *Rhodospirillum rubrum*, a membrane-bound PPase can function as a H^+-pump and the proton gradient can drive ATP synthesis [15]. For the first

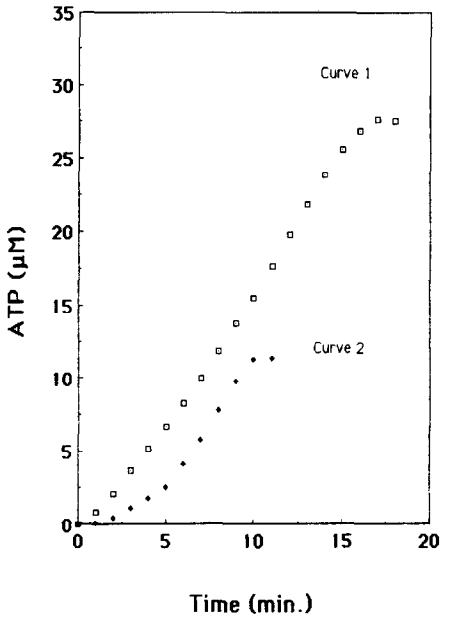

Fig.4. Time course of ATP synthesis in a suspension of tonoplast-enriched vesicles. Experimental conditions: 0.1 ml vesicles (200 μg) were suspended in 0.9 ml of 50 mM Hepes-BTP at pH 7.75 containing 0.25 M sorbitol, 10 mM $MgCl_2$, 50 mM KCl, 2 mM EDTA, 1 mM DTT, 0.15 mM AMP. (Curve 1) After 15 min incubation in the presence of 0.76 mM PP_i, the ATP synthesis was measured by adding 0.1 ml luciferin (0.3 mM)/luciferase (0.8 μg/ml) and 0.6 mM ADP, final concentrations, in the same buffer. (Curve 2) Same conditions as in curve 1 but without incubation.

time, we have demonstrated the existence of such energy-linked reactions located on tonoplast-enriched vesicles prepared from superior plant cells.

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