

This paper is dedicated to the memory of Professor Kazimierz Toczko
Communication

The effect of inorganic pyrophosphate on the transport of oleanolic acid monoglycosides into vacuoles isolated from *Calendula officinalis* leaves

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The influence of exogenous inorganic pyrophosphate on the transport of oleanolic acid monoglucoside and monoglucuronide to vacuoles isolated from *Calendula officinalis* leaf protoplasts was studied. The results indicate that the transport of both monoglycosides is carrier-mediated; however, the transport of the monoglucuronide is passive, and that of the monoglucoside active. The active transport of the monoglucoside is dependent on tonoplast energization created as a result of cooperation of two vacuolar proton pumps: H⁺-ATPase and H⁺-PPase.

The vacuolar membrane (tonoplast) of higher plant cells contains two functionally and physically distinct phosphohydrolases: a vacuolar-type H⁺-ATPase (EC 3.6.1.3) and a H⁺-PPase (inorganic pyrophosphatase, EC 3.6.1.1) [1-3]. These two enzymes catalyze the hydrolysis of ATP and PPi, respectively, and mediate electrogenic proton translocation into the vacuole, creating a low internal pH and an interior-positive membrane potential

(Fig. 1). Thus, the two proton pumps generate a transmembrane electrochemical potential, providing an energy source for the transport of various compounds into the vacuolar sap. However, the reason for the presence of the two enzymes in the same membrane remains unclear. It is also unknown how these two pumps cooperate and whether they are under independent cytoplasmic control.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; PCMBs, *p*-chloromercuribenzenesulfonic acid.

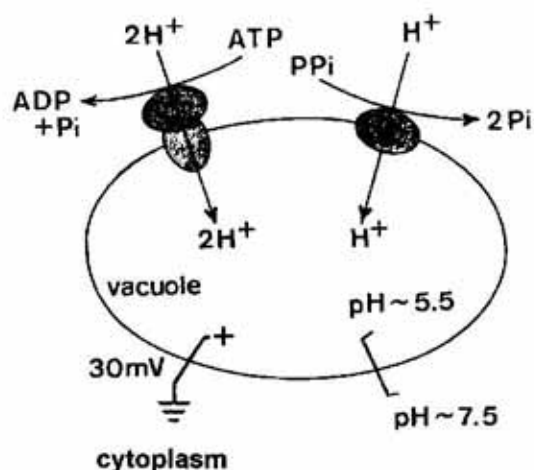


Figure 1. Scheme of tonoplast energization by two proton pumps: H^+ -ATPase and H^+ -PPase in vacuoles of higher plants.

Since the transport of various compounds into the vacuole has been intensively investigated in the last years, it is now obvious that the translocation across the tonoplast may be either a passive process, based on simple or facilitated diffusion, or an active, energy-dependent mechanism, driven by two vacuolar proton pumps mentioned above. For instance, we have demonstrated the tonoplast transport and vacuolar accumulation of oleanolic acid monoglycosides (Fig. 2) in *Calendula officinalis* leaves [4-6]. It was shown that the transport of monoglucuronide F, al-

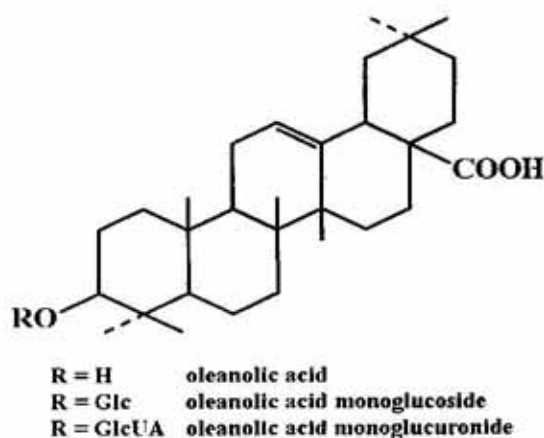


Figure 2. The structure of oleanolic acid and its monoglycosides present in *Calendula officinalis* leaves.

though specific and carrier-mediated, is passive and ATP-independent, whereas the transport of monoglucoside I is also carrier-mediated, but is an active and energy-requiring mechanism. The active transport of oleanolic acid monoglucoside has been well characterized with respect to its ATP-dependence [4, 6]. Therefore, the aim of the present studies was to investigate the effect of inorganic pyrophosphate on the oleanolic acid monoglycosides transport to the vacuoles isolated from *Calendula officinalis* leaf protoplasts.

MATERIALS AND METHODS

Isolation of protoplasts and vacuoles. Protoplasts were isolated from leaves of *C. officinalis* as described earlier [7]. Vacuoles were liberated from protoplasts with DEAE-dextran in isotonic conditions, then purified by centrifugation in a discontinuous mannitol-sucrose-Ficoll gradient and afterwards stabilized as described previously [5, 8].

Radioactive precursors. 3-O-Monoglucoside and 3-O-monoglucuronide of [$3\text{-}^3\text{H}$]oleanolic acid were chemically synthesized as described earlier [9]. The obtained labeled compounds had a specific activity of 3.82 mCi/mmol.

Administration of radioactive precursors. The incubation of isolated vacuoles with radioactive compounds (2×10^5 d.p.m./ 4×10^5 vacuoles in 1 ml of incubation medium [5]) was carried out at an illumination of 3000 lux and temperature of 25°C for 60 min. To determine the effect of inorganic pyrophosphate, the incubation procedures were carried out in standard conditions as described in [10] including 50 mM KCl and 1 mM MgSO_4 with $\text{Na}_4\text{P}_2\text{O}_7$ in 0.5-2.5 mM concentration range. To determine the inhibitor sensitivity of the monoglycosides transport, vacuoles were preincubated for 20 min with the inhibitor being tested in the presence or absence of 1.0 mM PPI, than the transport of radioactive mono-

glycoside was carried out. After the incubation, the labeled monoglycosides nonabsorbed into the vacuoles were washed off by centrifugation in the mannitol-sucrose-Ficoll gradient.

Radioactivity measurements. The fraction of intact vacuoles purified after the incubation was extracted with ethyl ether and *n*-butanol. Radioactivity of the monoglycosides taken up was estimated in a Beckman scintillation counter.

RESULTS AND DISCUSSION

The results concerning the dependence of oleanolic acid monoglycosides transport into isolated vacuoles on inorganic pyrophosphate are recorded in Figs. 3 and 4. It was found that both monoglycosides distinctly differ in the effect of exogenous PPI on their tonoplast transport. Namely, it was shown that PPI at 0.5–2.5 mM concentrations stimulated the transport of monoglucoside I (Fig. 3), whereas it exerted no effect on the transport of monoglucuronide F (Fig. 4). For comparison, the effect of ATP on the transport of the monoglucoside and the monoglucuronide, estimated from results obtained earlier [4], was also recorded in Figs. 3 and 4, respectively. It was shown that PPI as well as ATP stimulated the transport of the monoglucoside. However, the influence of PPI was strongest at 1 mM concentration, where the transport of the monoglucoside increased twofold, and that of ATP – at 1.5 mM, where over fourfold increase of the transport was noted (Fig. 3). In PPI concentrations higher than 2 mM a slight inhibitory effect was observed, whereas ATP caused transport reduction in concentrations higher than 5 mM (data not shown). On the contrary, neither PPI nor ATP exerted any effect on the transport of the monoglucuronide (Fig. 4). These results imply that active transport of monoglucoside I is dependent not only on ATP, but also on PPI, thus indicating the cooperation of both proton pumps, i.e. H^+ -ATPase and H^+ -PPase in gener-

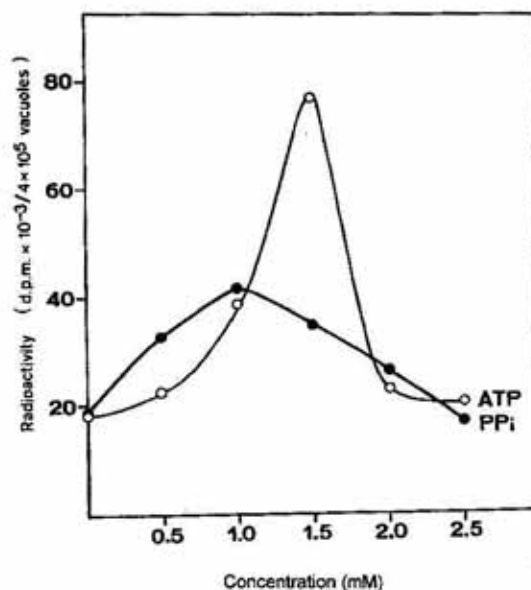


Figure 3. Effect of PPI (●) and ATP (○) on the transport of oleanolic acid monoglucoside to isolated vacuoles. The results are the mean values of four replicate experiments.

ating the proton motive force and membrane potential in *C. officinalis* vacuoles.

Further evidence in favor of the role of H^+ -PPase in the energization of active tonoplast

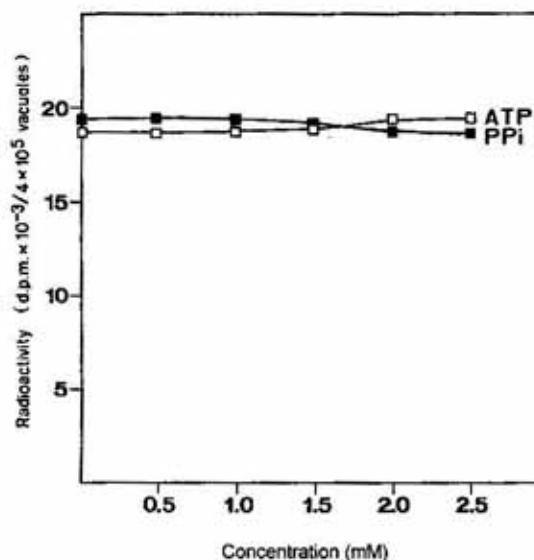


Figure 4. Effect of PPI (■) and ATP (□) on the transport of oleanolic acid monoglucuronide to isolated vacuoles.

The results are the mean values of four replicate experiments.

transport system is provided by the data in Table 1, concerning the effect of various inhibitors, i.e. uncoupling factors, protonophores and a protein-modifying agent. The influence of CCCP, DCCD, DIDS and PCMBS on the transport of both monoglycosides was tested in the absence or presence of 1 mM PPI. The results, summarized in Table 1, are mean values for at least three independent experiments. Again, it was found that both monoglycosides differ in the sensitivity of their transport to individual inhibitors. In general, the transport of monoglucoside I to isolated vacuoles was significantly reduced by uncouplers and ionophores as well as the protein-modifying agent. However, the inhibitory influence of CCCP and DIDS were higher in the absence (57% and 78%, respectively) than in the presence of PPI (27% and 14%). Indeed, all factors tested were not exclusive specific inhibitors of H⁺-PPase. CCCP, although regarded as an electrogenic H⁺ ionophore dissipating proton electrochemical gradient, is less effective towards H⁺-PPase than towards H⁺-ATPase, similarly as DIDS, known as an anion-channel blocker rather than being specific to tonoplast ATPase [11]. As we demonstrated earlier [6], CCCP and DIDS strongly reduced the transport of monoglucoside I in

the presence of ATP (88% and 93%, respectively). Therefore, the explanation of the observed effect may be, that one of the potential physiological roles of vacuolar H⁺-PPase is to act as a supplementary H⁺-pump constituting a back-up for the H⁺-ATPase in the case of its malfunction or under conditions when ATP availability is limiting [12]. In turn, DCCD reduced the transport of the monoglucoside by 79% in the absence and by 85% in the presence of 1 mM PPI. As we reported earlier [6], the inhibition of the transport of monoglucoside I by DCCD in the presence of ATP was also very high (95%). These results can be explained by the mechanism of inhibition by DCCD, which acts directly on proton channels of H⁺-ATPases as well as H⁺-PPases.

By contrast, CCCP, DCCD and DIDS exerted no effect on the transport of monoglucuronide F in the absence and presence of PPI as well as ATP, as we reported previously [6]. The only factor which strongly influenced the transport of both monoglycosides to isolated vacuoles was the protein-modifying agent PCMBS. The transport of the monoglucoside as well as the monoglucuronide was almost completely (96–98%) reduced by PCMBS in all conditions tested. The inhibitory influence of this compound, however, can be explained not only by

Table 1. Effect of various inhibitors on the transport of oleanolic acid monoglycosides into isolated vacuoles of *Calendula officinalis* leaf protoplasts

Inhibitor	Inhibitor concentration (mM)	Inhibition (%)			
		Monoglucoside I		Monoglucuronide F	
		Preincubated with			
		-PPI	+ PPI	-PPI	+ PPI
CCCP	0.05	28	20	1	1
	0.1	57	27	1	1
DCCD	0.02	23	75	0	1
	0.05	60	78	0	0
	0.1	79	85	1	2
DIDS	0.05	55	10	0	1
	0.1	78	14	2	3
PCMBS	0.1	88	87	86	86
	1	96	95	98	97

blocking the transport energization *via* H^+ -PPase (or H^+ -ATPase) inhibition, but mainly by direct action on membrane carriers, thus indicating carrier-mediated transport of both monoglycosides. In turn, transport of both monoglycosides was insensitive to orthovanadate (0% inhibition in all cases, not shown), which could be expected since this agent is most effective as an inhibitor of plasma membrane H^+ -ATPase and soluble alkaline phosphatase. It was also shown that nitrate, known as a tonoplast H^+ -ATPase inhibitor, significantly stimulated (30% at 100 mM of KNO_3) the transport of monoglucoside I in the presence of PPI, whereas, as we demonstrated earlier [6], it reduced this transport by 94% in the presence of ATP. This effect can be explained by K^+ -activation of tonoplast H^+ -PPase [13]. The fact that proton pumping by the H^+ -PPase exhibits an obligatory dependence on the presence of K^+ is well documented [13, 14]. Moreover, this observation raises the possibility that, rather than merely acting as an auxiliary H^+ -pump, the H^+ -PPase has its own distinct functions and, for instance, serves also to catalyze direct translocation K^+ of across the tonoplast [14, 15]. It would be another possible reason for the existence of two H^+ -pumps in the same membrane.

The results obtained in this work give a definite support to the concept that the transport of oleanolic acid monoglycosides to vacuoles isolated from *C. officinalis* leaf protoplasts varies in the mechanism of tonoplast translocation. The transport of monoglucuronide F is a passive and carrier-mediated process, whereas the transport of monoglucoside I is an active, carrier-mediated mechanism. The active mechanism of monoglucoside transport is dependent on tonoplast energization created by two vacuolar proton pumps: H^+ -PPase and H^+ -ATPase. Since the effect of ATP on the transport of the monoglucoside is twice stronger than the influence of PPI (Fig. 3), H^+ -ATPase can be regarded as the dominant pump and must be given the primacy in generating the tonoplast electrochemical potential.

However, the data obtained point to the possibility that under conditions of H^+ -ATPase malfunction or limited ATP availability, H^+ -PPase can act independently and provide a level of tonoplast energization enough to maintain the vacuolar active transport systems.

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