Zeitschrift: Archives des sciences et compte rendu des séances de la Société
Herausgeber: Société de Physique et d'Histoire Naturelle de Genève
Band: 49 (1996)
Heft: 2: Archives des Sciences

Artikel: Proton transport ATP dependent driven by the plasma membrane ATPase from Arabidopsis thaliana leaves : biochemical characterization

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DOI: https://doi.org/10.5169/seals-740421

Archs Sci. Genève	Vol. 49	Fasc. 2	pp. 159-171	Septembre 1996

Communication présentée à la séance du 8 février 1996

PROTON TRANSPORT ATP DEPENDENT DRIVEN BY THE PLASMA MEMBRANE ATPase FROM *ARABIDOPSIS THALIANA* LEAVES - BIOCHEMICAL CHARACTERIZATION

BY

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ABSTRACT

Proton transport driven by the plasma membrane ATPase from *Arabidopsis thaliana* **leaves. Biochemical characterization. -** The plasma membrane from *Arabidopsis thaliana* leaves was purified by phase partitioning and the use of enzyme markers showed that this fraction was highly enriched in plasma membrane. This fraction was almost devoid of phosphohydrolase activities originating from endomembranes (tonoplastes, mitochondria, non specific phosphatases and golgi apparatus). The H^+ATPase in this fraction was Mg^{2+} dependent and stimulated by K^+ and valinomycin. It was almost unsensitive to nitrate (tonoplaste ATPase inhibitor) but sensitive to vanadate (plasma membrane ATPase inhibitor) and other known ATPase inhibitors, especially the omeprazol inhibited both ATPase activity and plant growth. This activity was specific for ATP with a Kmapp of 392 µM and had a pH optimum around 6.7. On the other hand, 1 µM of lysophosphatidylcholine stimulated the H^+ transport activity into the purified plasma membrane vesicles. Higher concentration of this detergent (30 µM) was inhibitory.

Key words: *Arabidopsis thaliana*, Adenosine triphosphatase, H^+ pump, Omeprazol.

Abbreviations: ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; BSA, bovine serum albumin; BTP, bis-tris-propane (1,3-bis (tris (hydroxymethyl) methylamino) propane); DCCD, Dicyclohexylcarbodiimide; DTT, dithiothreitol; EB, Erythrosine B; GTP, Guanosine triphosphate; IAA, indole-3-acetic acid; IDPase, inosine diphosphatase; Mes, 4 morpholinoethane sulfonic acid; PEP, Phosphoenolpyruvate; PNPP, p-nitrophenylphosphate; PMSF, phenylmethyl-sulfonylfluoride; PP_i, Pyrophosphate; Tris, Tris (hydroxy methyl)-aminomethane; UDPG, uridine diphosphoglucose.

INTRODUCTION

The plasma membrane of cells plays a crucial role in the perception, transduction and transport of environnemental signals within the cell leading to a particular response. One of the important enzymes which seems to control growth, development, nutrient transport and stomata mouvements is the H^+ pump of the plasma membrane (SERRANO, 1989). This enzyme belongs to the P-type family of cation-translocating enzymes and generates an electric potentiel and pH gradient (H^+ motive force) that drive solute mouvements cross the plasma membrane.

The study of a particular cell response uses classic physiological and biochemical approaches with recently more and more integration by molecular-biological

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approaches. For this purpose the use of an experimental material which contains a small genome should be the most suitable. Among higher plants, *Arabidopsis thaliana* verify this condition but also have a small size, short generation time and large seed output. Moroever, many interesting mutants affected in different physiological functions are available, so that we can relate a response to a particular physiological function.

In this paper, we report the purification of the plasma membrane from *Arabidopsis thaliana* seedlings. The highly purified plasma membrane fraction suitable for biochemical studies was characterized by enzyme markers. The H^+ ATPase in this fraction was also characterized and the effect of many ATPase inhibitors was tested among them the omeprazol which is an anti-ulcer and inhibits the gastric H^+/K^+ ATPase by reacting to SH-groups of catalytic sites of the PM-bound ATPase (REMIS *et al.*, 1994; LINDBERGER *et al.*, 1990).

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana (L.) Landsberg erecta seeds were kindly provided by Dr. Maarteen Koornneef (Wageningen). Plants were cultivated in soil (Teramax Belflor, Bachman S. A., Chevroux, Switzerland) and grown in a phytotron for 4 weeks under non inductive short days (SD) of 8 h light (8:00 am to 4:00 pm) and 16 h dark (4:00 pm to 8:00 am). The temperature was set at 20 ± 1°C and the relative humidity at 80 ± 5 % during the light period and 60 ± 5 % during the darkness. The light was provided by white fluorescent tubes (40 W, 244332, 400 mol m^{-2} s^{-1}, Sylvania daylight, USA).

Preparation of microsomal vesicles

Crude microsomal vesicles were prepared as described by OLIVARI *et al.* (1993). Leaves (20 g) were harvested and homogenized at 4°C in a medium (4 ml per g fresh weight) containing 250 mM sucrose, 10 mM EGTA, 2 mM MgSO4, 2 mM Na_2 ATP, 1 mM PMSF, 2 mM DTT, 0.5 % (w/v) BSA, 25 mM BTP-MES, adjusted to pH 7.6 in the presence of polyclar (1 g / 20 g fresh weight). The homogenate was filtered and centrifuged at 6,000 x g (7,000 rpm, Sorvall, SS-34 rotor) for 10 min at 4°C. The resulting supernatant was filtered through one layer of Miracloth (475855 Calbiochem) and centrifuged at 30,000 x g for 30 min at 4°C. The pellet (crude microsomes) was resuspended in phosphate buffer (KH_2PO_4: Na_2HPO_4, 5 mM, pH 7.8) and plasmalemma was purified by phase system containing 6.2 % PEG, 6.2 % Dextran, 0.44 mM potassium phosphate buffer pH 7.8, 3 mM KCl and 0.22 M sucrose (KJELLBOM & LARSSON, 1984). Upper phases U3 and U'3 were combined and plasma membrane vesicles were recovered by centrifugation at 30,000 x g for 30 min at 4°C, washed in BTP-Mes 25 mM pH 6.7 additioned with 10 mM EGTA, resuspended in the same medium without EGTA and used immediatly. For the test of the H^+pumping activity, the plasma membrane vesicles recovered by centrifugation were washed in the resuspension medium (250 mM sucrose,

10 % glycerol, 1 mM DTT, 0.2 % BSA, 1 mM PMSF, 2.5 mM BTP-MES pH 7.0) additioned with 10 mM EGTA, resuspended in the same medium without EGTA, and stored at - 80°C until used.

Proton pumping activity

The initial rate of quinacrine fluorescence quenching was utilized to measure the plasmalemma proton translocation activity as described by BENNET & SPANSWICK (1983). This activity was expressed in arbitrary unit (AU) min^{-1} mg^{-1} protein. Fluorescence was measured with an Aminco Bowmann spectrofluorimeter at the excitation/emission wavelengths of 420/500 nm. Thawed membrane vesicles (20-30 µg proteins) were incubated at room temperature for 10 min. in 1 ml of 25 mM BTP-Mes pH 6.7, 2 µM quinacrine, 0.25 µM valinomycin, and 50 mM KBr. The reaction was started by addition of 1.5 mM Mg ATP. The quenching of quinacrine fluorescence was completely reversed by addition of 10 µl of 1 mM monensin. The reaction could also be started by addition of 1.5 mM Na_2 ATP, in this case the reaction medium contained 5 mM $MgSO_4$.

ATPase activity

The plasmalemma ATPase activity was measured at 37°C during 30 min in the presence of 50 mM KCl, 5 mM $MgSO_4$, BTP-Mes 25 mM pH 6.7. The reaction was started by addition of 1.5 mM Na_2 ATP. If Mg ATP had to be used $MgSO_4$ was omitted from the reaction medium. The membrane vesicles were pelleted at 12,000 rpm (microfuge Sorvall) for 10 min at 4 °C and the inorganic phosphate released by ATP hydrolysis was determined by adding 0.2 ml of the resultant supernatant to 0.5 ml of 0.24 % (w/v) ammoniummolybdate (in 0.5 M H_2SO_4), 0.96 % (w/v) ascorbic acid and 0.8 % (w/v) trichloroacetic acid. After incubation at 36 °C for 10 min the absorbance at 660 nm was measured. To measure the latency activity triton X100 was added at a triton to protein ratio of 10 (µg/µg). At this concentration the ATPase activity was the highest.

Other assays

Glucan synthase II was determined after KAUSS & JEBLICH (1985). UDPG sterol-glycosyl transferase was determined after CHANSON *et al.* (1984). IDPase latency was determined after NAGAHASHI & HIRAIKE (1982). NADH dependent cytochrome C reductase was determined after PALMGREN *et al.* (1990). Cytochrome C oxidase was determined after HODGES & LEONARD (1972). Pyrophosphatase activity was determined after CHANSON & PILLET (1988).

Protein determination

An aliquot (30 µl) of membrane preparation was diluted 20 fold with cold water and centrifuged at 96,100 x g (40,000 rpm, T-50 rotor, Beckman) for 30 min at 5°C to remove BSA from the resuspension medium. The pellet was resuspended in cold water (160 µl) and proteins were determined using the Bio-Rad protein assay solution with BSA as the standard.

RESULTS AND DISCUSSION

Membrane vesicles prepared by aquous phase partitioning were characterized by plasma membrane markers (glucan Synthase II, UDPG sterolglycosyltransferase, proton pumping ATP dependent) and by other markers (NADH-cytochrome reductase for endoplasmic reticulum, NADH-cytochrome C oxidase for mitochondria, IDPase activity for Golgi, pyrophosphatase for tonoplast). As shown in table 1, the fraction of the combined upper phases U_3 and U'_3 was highly enriched in plasma membrane vesicles. The plasma membrane markers were more present in the combined phases (U_3 and U'_3) than in the lower phase L, and the endomembranes markers were more present in the lower phase L than in the combined phases (U_3 and U'_3). The NADH-cytochrome C reductase may serve as a marker for the endoplasmic reticulum preparation, but the plasma membrane also contains this activity (WIDELL & LARSSON, 1990). The obtained results indicated that the plasma membrane fraction was more enriched in the NADH-cytochrome C reductase activity than the endomembranes fraction. This because the results were expressed in specific activities than total activities and seem to be similar to that found by LARSSON *et al.* (1987).

TABLE 1

Characterization of plasma membrane purified by two phase partitioning: Enzyme markers of membranes present in upper phase (U_3+ U'_3) and lower phase (L) after two-phase partitioning of a crude microsome preparation from *Arabidopsis thaliana* leaves. U_3 + U'_3 represents the purified plasma membrane fraction. L phase represents the endomembranes fraction. The values betwen parenthesis represent the enrichment relative to the specific activities of the microsomal fraction.

Specific activity of enzyme markers	Crude microsomes	U_3+U'_3	L
Glucane synthase II (Dpm min^{-1} mg^{-1} prot.)	33404	91174 (2.73)	20198 (0.60)
UDPG-sterol transferase (Dpm min^{-1} mg^{-1} prot.)	609	2462 (4.04)	303 (0.50)
NADH dep.cyt.C reductase (nmol min^{-1} mg-1 prot.)	96	212 (2.21)	106 (1.10)
ATP dependent H^+transport (AU min^{-1} mg^{-1} prot.)	nd	41.2 (--)	nd
Pyrophosphatase (AU min^{-1} mg^{-1} prot.)	nd	9.8 (--)	nd
ATPase activity (µmol Pi min^{-1} mg^{-1} prot.)	0.199	0.677 (3.40)	0.283 (1.42)
IDPase activity (µmol Pi min^{-1} mg^{-1} prot.)	0.161	0.015 (0.09)	0.159 (0.99)
Cyt.C oxidase (nmol min^{-1} mg^{-1} prot.)	39	0 (0)	40 (1.03)

nd: not determined.

The method used for plasma-membrane purification was reported by many authors (LARSSON *et al.*, 1988, PALMGREN *et al.*, 1990) to give rather right side out plasma-membrane vesicles. The ATPase latency which corresponds to the proportion of the right side out vesicles in the plasma membrane fraction, was about 70 %. The ATPase activity of the purified plasma membrane showed a pH optimum arround 6.7 (Fig. 1), similar to that described for other plant species (DE MICHELIS & SPANSWICK, 1986; OLIVARI *et al.*, 1993; BELLAMINE & GREPPIN, 1994). The plasma membrane ATPase

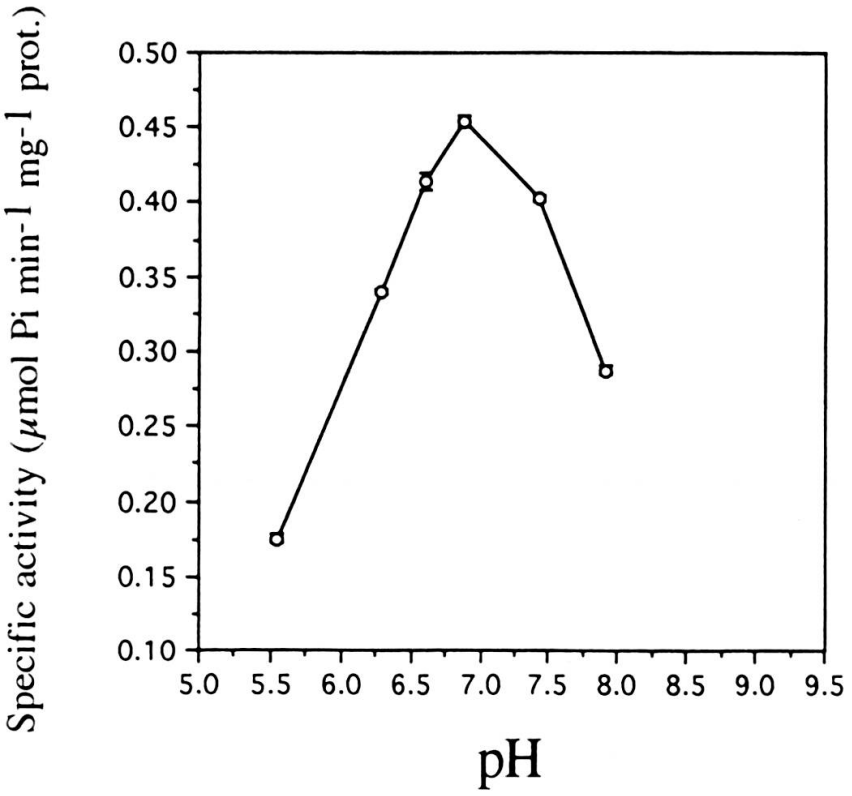

FIG. 1

pH effect of the reaction medium on the ATP hydrolase activity of the purified plasma membrane. The pH of the medium was adjusted by varying the proportions of BTP and Mes (50 mM final concentration). The reaction medium included 50 mM KCl. The reaction was started by addition of 1.5 mM Mg ATP.

from *Arabidopsis thaliana* leaves requires rather ATP as source of energy than other phosphorylated substrats tested (Fig. 2). The apparent Michaelis-Menten constant (Km_{app}) for ATP was 392 μM and V_{max} of 0.584 μmol P_i min^{-1} mg^{-1} protein (Fig. 3), similar to that found for other plant species (RASI-CALDOGNO *et al.*, 1985; DE MICHELIS & SPANSWICK, 1986; BELLAMINE & GREPPIN, 1994).

The purified plasma membrane fraction was assayed for proton pumping activity in the presence of different substances. This activity was Mg^{2+} dependent, and was stimulated by K^+ and by valinomycin (Tab.2). The plasma membrane proton pumping activity was 6 % inhibited by 50 mM KNO_3 (vacuolar ATPase inhibitor) and 53 % inhibited by only 200 μM vanadate (plasma-membrane ATPase inhibitor) (Tab. 3).

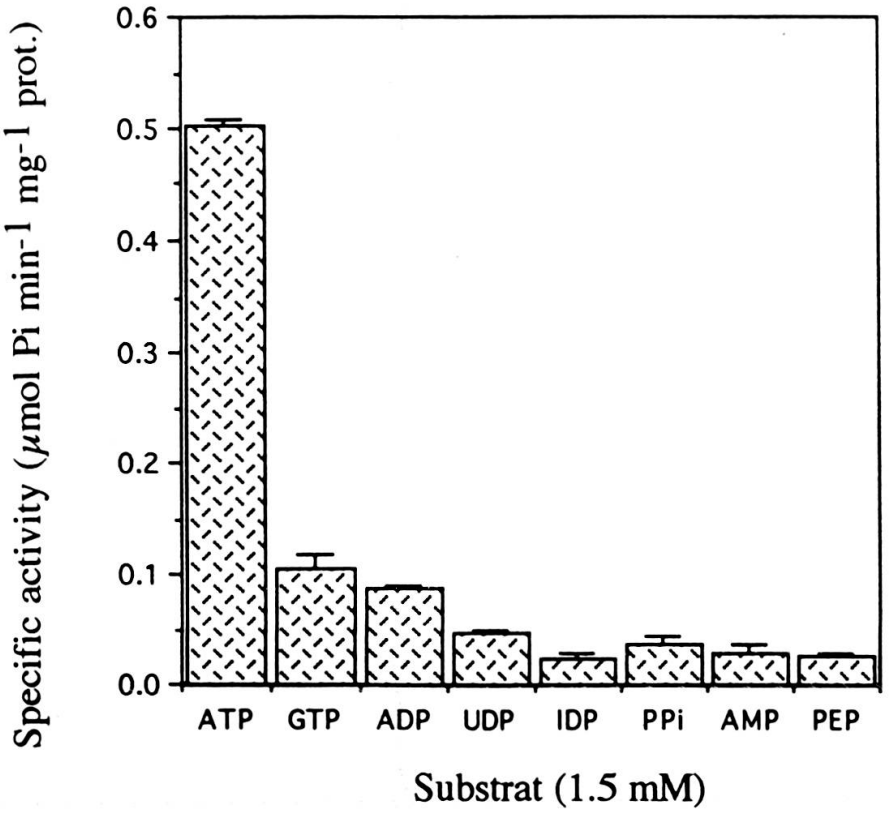

FIG. 2

Effect of some phosphorylated substrats on ATP hydrolase activity of the purified plasma membrane. Plasma membrane vesicles were incubated in the presence of 50 mM KCl and 5 mM $MgSO_4$ and the reaction was started by addition of 1.5 mM of the substrat. All the substrats were disodium salt except for phosphoenolpyruvate which was tricylohexylammonium salt.

TABLE 2

Proton pumping activity of the purified plasma membrane was tested for the effect of valinomycin, potassium and magnesium ions as described in Materials and Methods. The results are expressed in percent of the specific activity (AU min^{-1} mg^{-1} protein) to the control. The reaction was started by addition of 1.5 mM Mg ATP. When Mg^{2+} effect was tested, Mg ATP was replaced by Na_2 ATP.

Incubation medium	% H^+pumping activity to the control
Control	100
- Mg^{2+}	0
- Valinomycin	58
- KBr	35

TABLE 3

Proton pumping activity of the purified plasma membrane was tested in the presence of 50 mM KNO_3 (in this case the KCl was omitted) and 200 μM Vanadate. The reaction was started by addition of 1.5 mM Mg ATP and the results were expressed in percent of the specific activity to control (68.6 ± 0.97 AU min^{-1} mg^{-1} protein).

	Proton pumping activity % to the control ± SD
Mg ATP	100
KNO_3 (50 mM)	94 ± 3 %
Na_3VO_4 (200 μM)	37 ± 4 %

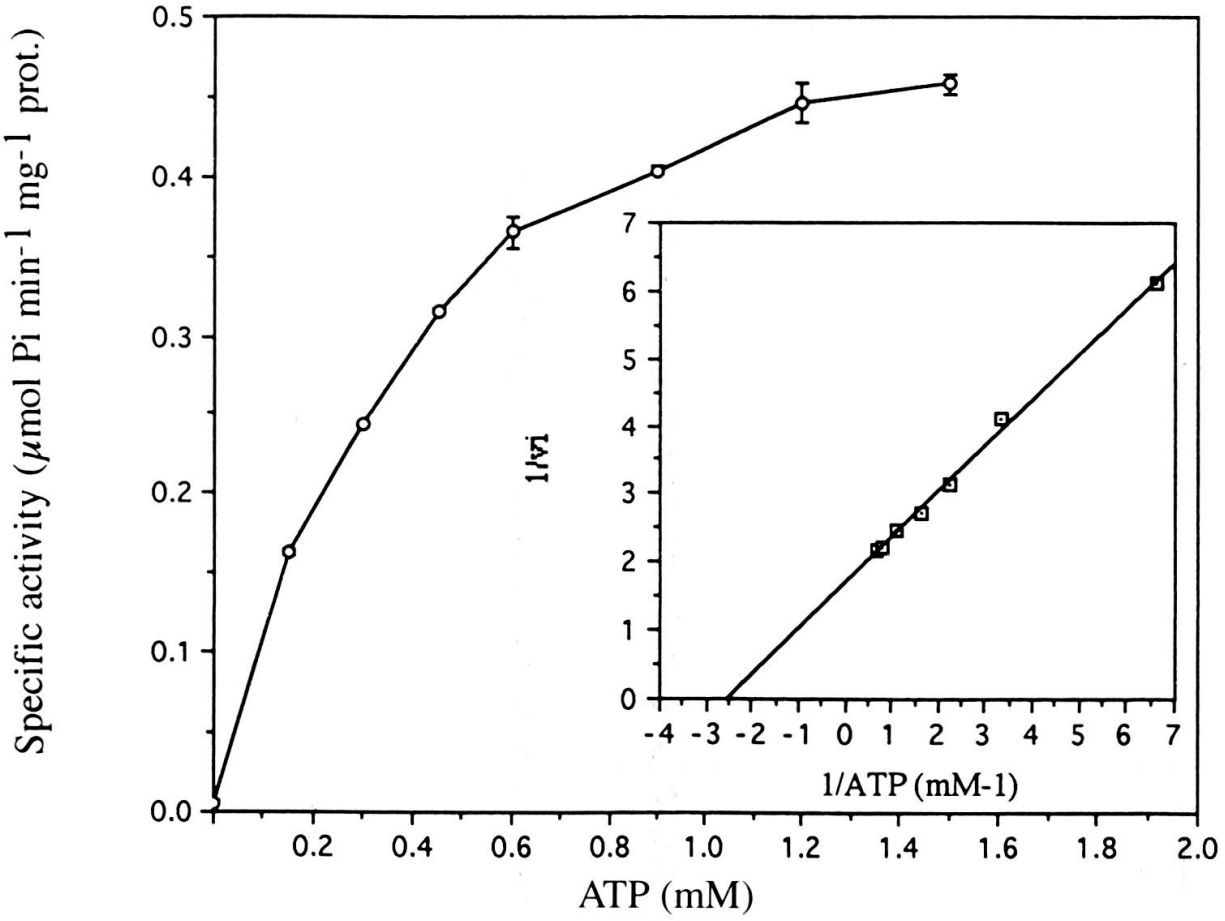

FIG. 3

$Km_{app.}$ determination for the ATP hydrolase activity of the purified plasma membrane. Insert shows Lineweaver-Burk double reciprocal plot of values. Plasma membrane vesicles were incubated in the presence of 50 mM KCl and 5 mM $MgSO_4$ and the reaction was started by addition of the indicated concentration of Na_2 ATP.

The ATPase-Mg dependent activity of the purified plasma membrane was almost unsensitive to nitrate, slightly diminished by 1 mM Na-molybdate (non specific phosphatase inhibitor) and by 5 mM azide (mitochondrial ATPase inhibitor), and showed that the purified plasma membrane fraction was almost devoid of phosphohydrolase activities originating from endomembranes as assessed by the effects of nitrate (tonoplast), azide (mitochondria), sodium molybdate and p-nitrophenylphosphate (non-specific phosphatases) or IDP (golgi apparatus) (Tab. 4). On the other hand the pyrophosphatase activity was almost absent in this fraction (Tab.1).

The purity of the plasma membrane fraction was determined by assaying biochemical marker enzymes for plasma membrane and endomembranes and showed that the fraction was highly plasma membrane enriched (Tab. 1). All the properties of the plasma membrane ATPase from *Arabidopsis thaliana* leaves (Mg^{2+} dependency, K^+ stimulation, specificity for ATP, optimum pH for activity, insensitivity for nitrate and sensitivity for vanadate) were similar to that found for the plasma membrane ATPase

activity from other plant species (BENNET & SPANSWICK, 1983; RASI-CALDOGNO *et al.*, 1985; DE MICHELIS & SPANSWICK, 1986; OLIVARI *et al.*, 1993; BELLAMINE & GREPPIN, 1994), and showed that the ATP hydrolysis and the proton transport into the purified vesicles were mostly due to the plasma membrane ATPase activity. Thereby, the decrease in the observed activity in the presence of some inhibitors will mostly due to the inhibition of the plasma membrane ATPase activity.

The ATP hydrolase activity of the purified plasma membrane fraction was assayed in the presence of different ATPase inhibitors. All of them are potent inhibitors of the plasma membrane ATPase from *Arabidopsis thaliana* leaves (Tab. 5), with more effective inhibition obtained for EB, DCCD and vanadate than for sodium fluoride. The EB have been reported to be a powerful inhibitor of rat brain synaptosomal Na-K-ATPase and of sarcoplasmic Ca-Mg-ATPase (MORRIS *et al.*, 1982). It inhibits also both the vanadate and the nitrate-sensitive ATPases in microsomal preparations from radish seedlings (COCUCCI, 1986 and references therein). A physiological concentration of fluoride as 10 mM inhibits the plasma membrane ATPase of *Arabidopsis thaliana* by about 70 % (Tab. 5), a result similar to that found for the plasma membrane ATPase activity from sugarbeet. Fluoride, a common air polluant long known as a toxicant to many plant processes, inhibits also mitochondrial, chloroplast and tonoplast ATPases, but at a higher than the physiological concentration usually used to inhibit the plasma membrane ATPase (GIANNINI *et al.*, 1987). This suggest that an initial effect of fluoride toxicity *in vivo* could occur at this site.

Among these inhibitors, the omeprazol inhibted the plasma membrane ATPase activity from *Arabidopsis thaliana* and from spinach leaves (Tab. 6) as demonstrated for the plasma membrane H^+ ATPase from acid resistant unicellular green alga Dunaliella acidophilia (REMIS *et al.*, 1994).

When spinach plant seedlings were grown in short day in the presence of omeprazol they developped a primary hairy root and smaller and more greening leaves probably by increasing the intracellular chlorophyll content. The petiole was almost absent in these plants (Fig. 4). It seems that the omeprazol is more effective than

TABLE 4

ATP hydrolysis activity was assayed in the presence of 50 mM KCl, 5 mM $MgSO_4$ (control) or with the modificaions indicated in the table. When the effect of KNO_3 was tested, the KCl was omitted. The reaction was started by addition of 1.5 mM ATP, PNPP or IDP. All the substrats were sodium salt. The results were expressed in specific activity ± SD (µmol Pi min^{-1} mg^{-1} protein). Values between barkets represent the percent of the activity to the control.

Treatment	ATP hydrolase activity ± SD (µmol Pi min^{-1} mg^{-1} protein)
Control	0.403 ± 0.0016 (100)
- Mg^{2+}	0.034 ± 0.0032 (8)
+ KNO_3 (50 mM)	0.373 ± 0.0042 (93)
+Sodium molybdate (1 mM)	0.365 ± 0.0052 (91)
+NaN_3 (5 mM)	0.402 ± 0.0040 (100)
PNPP (1.5 mM)	0.010 ± 0.0003 (2)
IDP (1.5 mM)	0.011 ± 0.0007 (2.7)

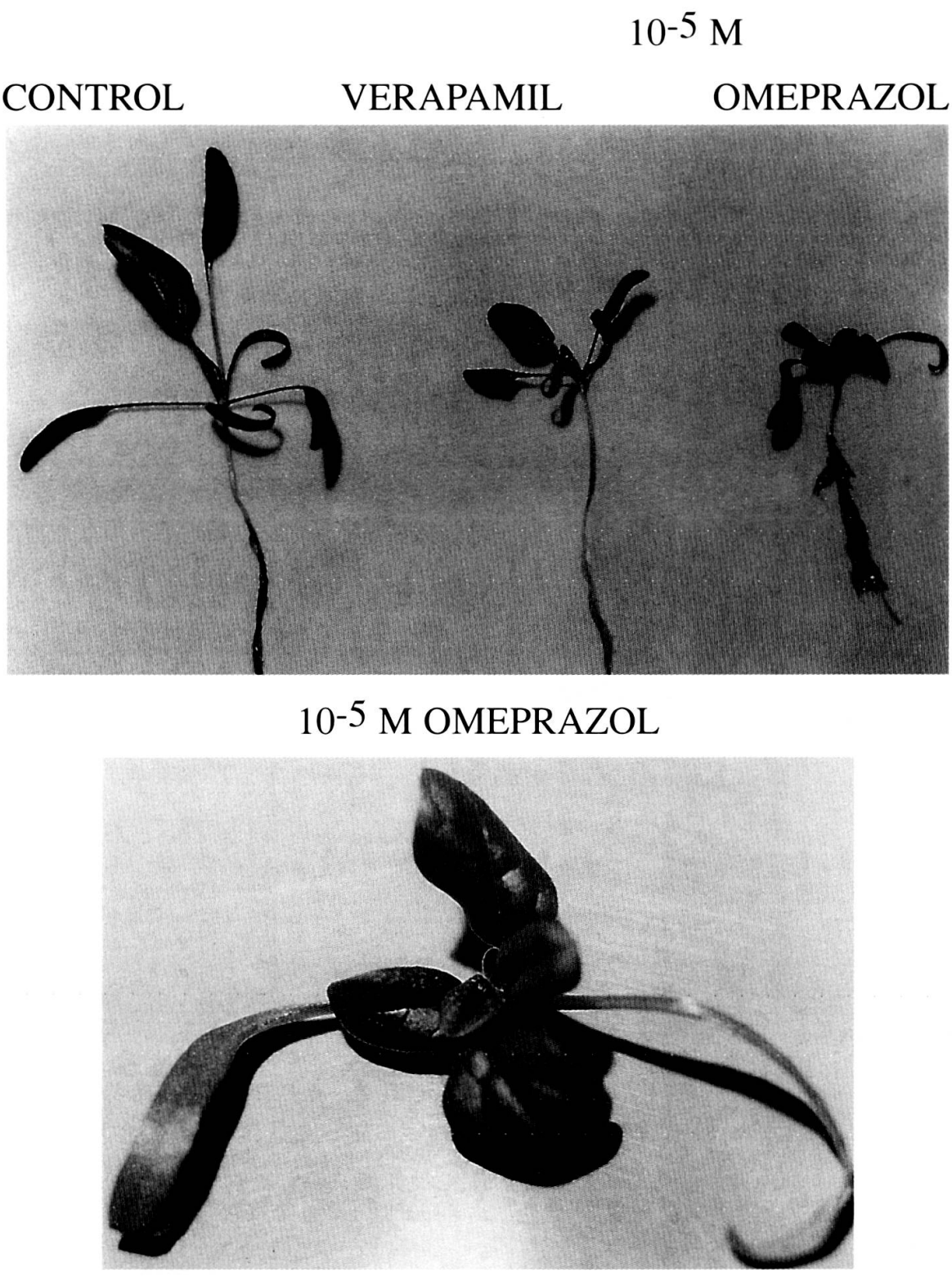

FIG. 4

Hydroculture in short days of 8 h of spinach plants on a nutritive medium (0.3 % Sinesol) in the absence (control) or in the presence of 10^{-5} M verapamil or Omeprazol 10^{-5} M. The photos were taken after 6 weeks.

TABLE 5

ATP hydrolysis activity was assayed in the presence of some inhibitors known to inhibit ATPases. The reaction medium included 50 mM KCl, 5 mM $MgSO_4$ and other substances indicated in the table. The results were expressed in specific activity ± SD (μmol Pi min^{-1} mg^{-1} protein). The reaction was started by addition of 1.5 mM Na_2 ATP. Values between barkets represent the percent of the activity to the control.

Inhibitors	ATP hydrolase activity ± SD (μmol Pi min^{-1} mg^{-1} protein)
Control	0.557 ± 0.0039 (100)
EB 10 μM	0.265 ± 0.0061 (48)
NaF 10 mM	0.176 ± 0.0046 (32)
DCCD 500 μM	0.107 ± 0.0140 (19)
$Na3VO_4$ 200 μM	0.140 ± 0.015 (25)
2 mM	0.100 ± 0.0012 (18)

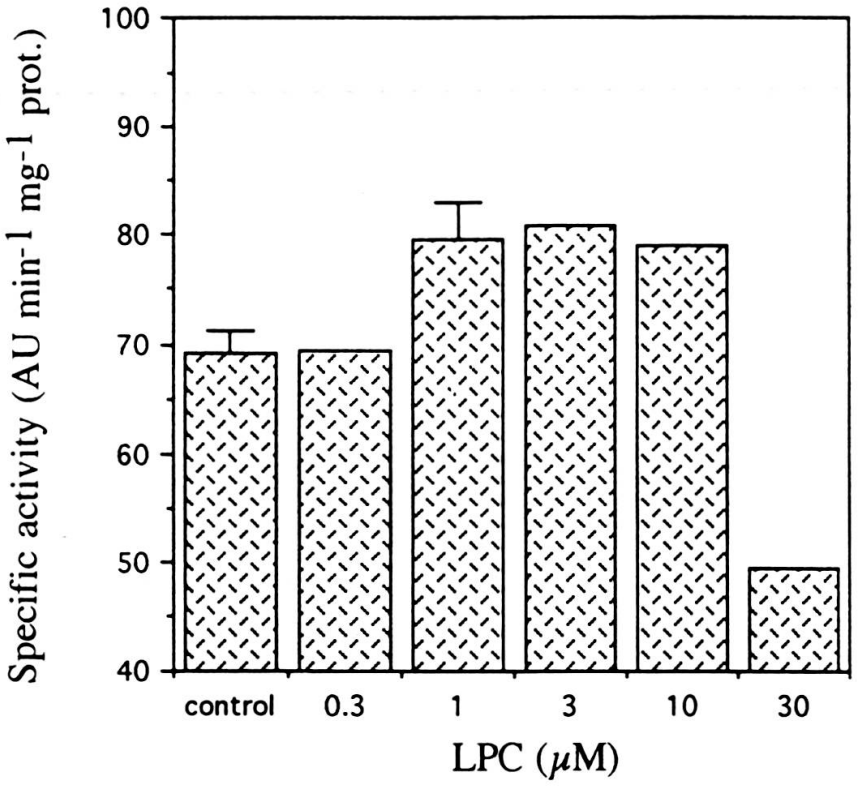

FIG. 5

Effect of lysophosphatidylcholine on the H^+ pumping activity of the purified plasma membrane. The plasma membrane vesicles were incubated 10 min in the reaction medium in the presence of the detergent and the reaction was started by addition of 1.5 mM Mg ATP.

TABLE 6

Effect of the omeprazol on the ATPase activity of the purified plasma membrane from *Arabidopsis thaliana* and from spinach leaves. The omeprazol was added to the reaction medium from 100 times concentrated solution in ethanol (control included 1 % ethanol). The results were expressed in specific activity ± SD (µmol Pi min^{-1} mg^{-1} protein). Values between barkets represent the percent of the activity to the control.

	ATP hydrolase activity ± SD (µmol Pi min^{-1} mg^{-1} protein)	
	Arabidopsis th.	*Spinach*
Control	0.603 ± 0.013 (100)	0.307 ± 0.002 (100)
+ Omeprazol:		
145 M	--	0.279 ± 0.002 (91)
290 M	0.453 ± 0.004 (75)	0.240 ± 0.001 (78)
435 M	0.434 ± 0.003 (72)	--

verapamil in inhibiting spinach growth. This could suggest the role of the plasma membrane ATPase in the plant growth and development and so emphasizes the acid growth theory of RAYLE & CLELAND (1992).

On the other hand, the H^+ transport activity was slightly (15 %) but significantly stimulated by 1 µM of lysophosphatidylcholine. Higher concentration (30 µM) of this detergent was inhibitory (Fig. 5). The plasma membrane H^+ ATPase could be stimulated by lysophospholipids either exogenously added to the plasma membrane reaction medium (PALMGREN & SOMMARIN 1989) or released *in situ* by a phospholipase A_2. In this case the auxin seems to activate the phospholipase activity (SCHERER & ANDRÉ 1993).

ACKNOWLEDGMENTS

We would like to thank ASTRA company (Sweden) for its kind gift of omeprazol.

RÉSUMÉ

La membrane plasmique des feuilles d'*Arabidopsis thaliana* a été purifiée par partition de phase et l'utilisation des marqueurs enzymatiques ont montré que cette fraction est hautement enrichie en membrane plasmique. Cette fraction est dépourvue des activités phosphohydrolases originaires des endomembranes (tonoplaste, mitochondrie, phosphatases non spécifiques et appareil de golgi). L' activité du transport de protons dans cette fraction est Mg^{2+} dépendante et est stimulée par le K^+ et la valinomycin. Elle est presque insensible au nitrate (inhibiteur de l'ATPase du tonoplaste) mais sensible au vanadate (inhibiteur de l'ATPase du plasmalemme) et à d'autres inhibiteurs connus des ATPases, en particulier l'oméprazole qui inhibe à la fois l'activité ATPase et la croissance végétale. Cette activité est spécifique pour l' ATP avec un Km_{app} de 392 µM et posséde un pH optimum de 6.7. La lysophos-

phatidylcholine (1 µM) stimule l'activité du transport de protons dans les vésicules de la membrane plasmique des feuilles *Arabidopsis thaliana*. Les plus fortes concentrations de ce détergeant sont inhibitrices.

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