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FURTHER EVIDENCE FOR THE PROTON PUMPING WORK OF TONOPLAST ATPase FROM HEVEA LATEX VACUOME

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An ATP-dependent acidification of internal medium of Hevea tonoplast vesicles could be monitored by 9-amino-6-chloro-2-methoxy-acridine. The reported data indicate a lack of effect of typical plasmalemma ATPase inhibitors (DES, vanadate) on the proton pumping activity of Hevea tonoplast ATPase.

A role of this tonoplast ATPase is evoked in the regulation of cytoplasm pH.

INTRODUCTION

Despite the obvious role of the vacuole of higher plant cells in the uptake and the accumulation of solutes, little is known about the movement of electrolytes and non-electrolyte molecules across the tonoplast.

Studies of the transport of citrate have been carefully studied with isolated vacuoles of Hevea latex (1) and with Hevea tonoplast vesicles(2). A transport against a transmembrane concentration gradient was found. Such a process is usually energized in eukaryotic cells by coupling the movement of solutes to the electrogenic transport of a cation, such as H^+ or Na^+ (3,4,5). Thus, the energy source is the difference in the electrochemical potential of cation which is usually generated by an electrogenic ion-translocating ATPase.

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In this paper, we reported experiments which showed that the MgATP-dependent acidification of internal medium of Hevea tonoplast vesicles could be monitored by fluorescent dyes as 9-amino-6-chloro-2-methoxy-acridine (ACMA). Consequently, we confirmed the results described elsewhere and obtained by different methodological means (2,6,7,8).

MATERIAL AND METHODS

1) Preparation of Hevea tonoplast vesicles

Tonoplast vesicles were prepared from Hevea latex as previously described (9).

2) Fluorescence measurements

Fluorescence measurements were conducted at room temperature with an Eppendorf spectrofluorimeter (excitation 405-436 nm ; emission 470-500 nm) according to Schairer et al. (10). The basic reaction mixture contained in 2.1 ml 25 mM M.O.P.S., 25 mM M.E.S., sufficient Tris-base to adjust pH to 7.5, 300 mM mannitol, 5 mM β-mercaptoethanol, 2.5 µM ACMA and tonoplast vesicles (about 250 µg protein). The sensitivity of the spectrofluorimeter was adjusted so that the addition of ACMA gave full scale deflection on the recorder. The ATP-dependent quenching of fluorescence was measured after the addition of 0.5 mM ATP and 0.6 mM $MgSO_4$.

3) Reagents

All reagents were of the purest grade available. Na_2ATP was obtained from Boehringer Mannheim. Carbonyl-cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) and ionophore 1799 were kindly given by Dr P.G. Heytler (Du Pont and Co.). Nigericin was a gift of Dr. R. Hamill (Eli Lilly and Co., Indianapolis, U.S.A.). ACMA was generously provided by Dr. H.V. Schairer.

RESULTS

Acridine compounds have been used as ΔpH probes to monitor the energization of membrane vesicles issued from different material (11, 12, 13). The mechanism of quenching of the probe is explained by a proton gradient induced distribution of the dye through the membrane.

Figure 1 shows that addition of MgATP to a suspension of Hevea tonoplast vesicles leads to a decrease of fluorescence. This Mg-ATP-dependent quenching is not affected by diethylstilboestrol (DES) and vanadate at the used concentrations (respectively) 0.9 mM and 0.09 mM). We confirme that these two well-known inhibitors of the

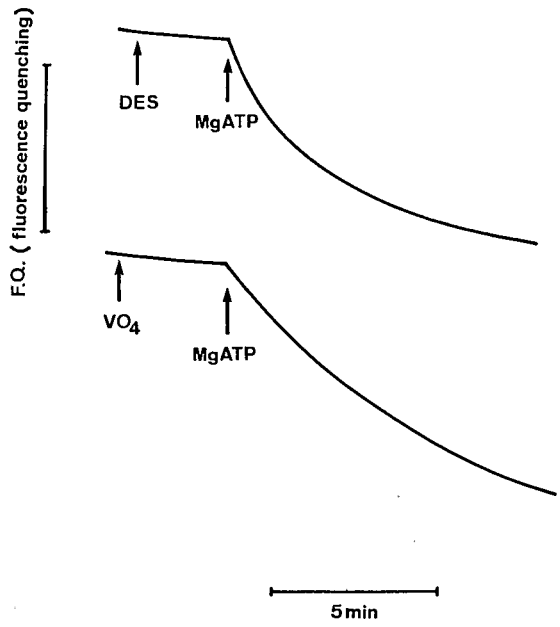

Figure 1. Effect of plasmalemma ATPase inhibitors on Hevea tonoplast ATPase activity.

Vesicles were incubated at room temperature in 2.1 ml standard medium. When indicated, additions were as follows : 10 µl D.E.S. (10^{-1}M) ; 10 µl VO_4 (10^{-2} M) and 10 µl Mg-ATP solution (ATP 10^{-1}M and Mg^{2+} 1.2 x 10^{-1}M).

Vesicles corresponded to a final concentration of 250 µg protein x ml^{-1}).

ATP-dependent proton pump of plasmalemma (14-17) have no effect on Hevea tonoplast ATPase which acts also as a proton pump (9). Consequently, these compounds do not affect not only the ATP hydrolysis catalyzed by the Hevea tonoplast ATPase but also the proton pumping activity of this membraneous enzyme.

FCCP is a protonophore which collapses with some difficulties the transmembrane proton gradient established through the Hevea tonoplast by the ATPase (7,8). This fact is confirmed by the results illustrated by Figure 2. When the ionophore is added after the fluorescence change had become constant, the subsequent increase of fluorescence is limited and never reaches the level of the initial fluorescence (Figure 2-A). However, if tonoplast vesicles were preincubated with FCCP before the addition of Mg-ATP, no

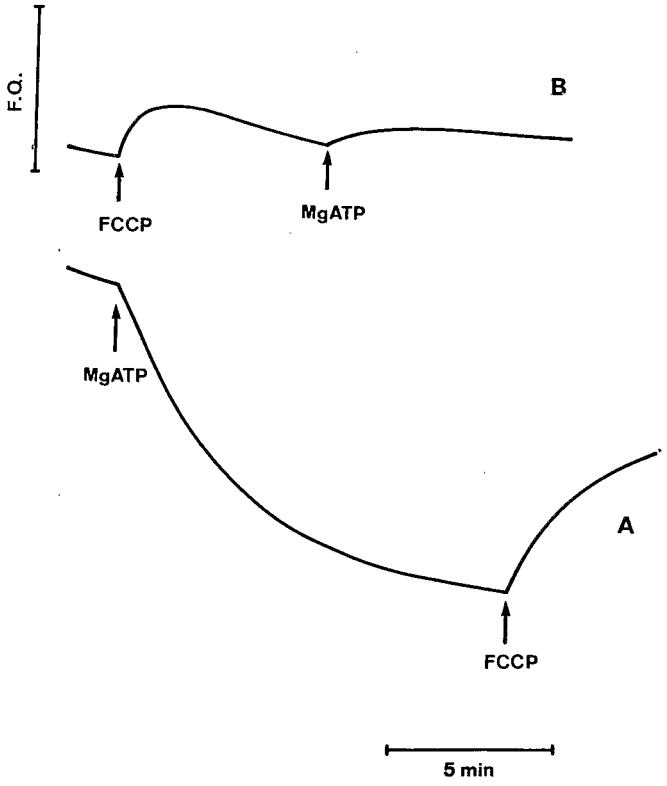

Figure 2. Effect of FCCP on Hevea tonoplast ATPase activity.

Experimental conditions were indentical to those in Figure 1. In the two runs, FCCP (10^{-3} M) and Mg ATP (5.10^{-3}M) were added in different order.

change in fluorescence intensity is observed. Such a result is in favour of an electrogenicity of ATPase activity in addition of the proton pumping activity. Moreover, some Donnan effect could be concerned in a preexisting tonoplast ΔpH as evoked elsewhere (6,7,8). The ionophore 1799 collapses the ATP-induced fluorescence changes in all the cases illustrated by Figures 3-A and 3-B. The difficulties met with FCCP are suppressed, as all the ATP-induced fluorescence is concerned by the ionophore action, the ionophore being added before or after the nucleotide.

In the presence of KCl, nigericin returns the fluorescence response to the original level (Figures 4-A and 4-B), as the H^+/K^+ exchange catalyzed by nigericin diminishes the acidification

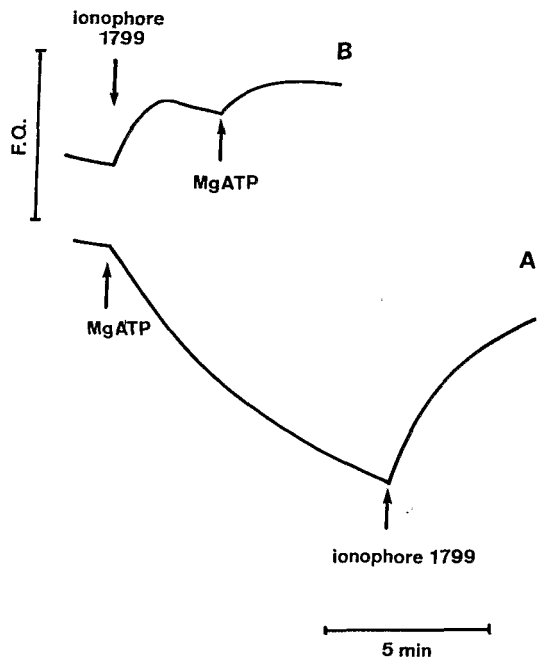

Figure 3. Effect of ionophore 1799 on Hevea tonoplast ATPase activity.
Experimental conditions were indentical to those in Figure 1. 5-10 µl of ionophore 1799 solution (5 mg x ml^{-1}) was added as indicated.

built up by the tonoplast ATPase. Valinomycin is without effect, confirming its absence of action on the fluorescent ΔpH probe behavior (Figure 4-B).

DISCUSSION AND CONCLUSION

The occurrence of an ATPase activity at the tonoplast level in higher plants was always debatable, since the work of I.J. METTLER and R.T. LEONARD in 1979 (18) and J. D'AUZAC (19). As described elsewhere (2,6-9), Hevea tonoplast ATPase works as a proton pump. The hydrolysis of ATP which takes place at the tonoplast level leads to an acidification of the intravacuolar medium and to a depolarization of tonoplast.

The acidification of internal medium of Hevea vacuoles and tonoplast vesicles was monitored either by accumulation of methylamine

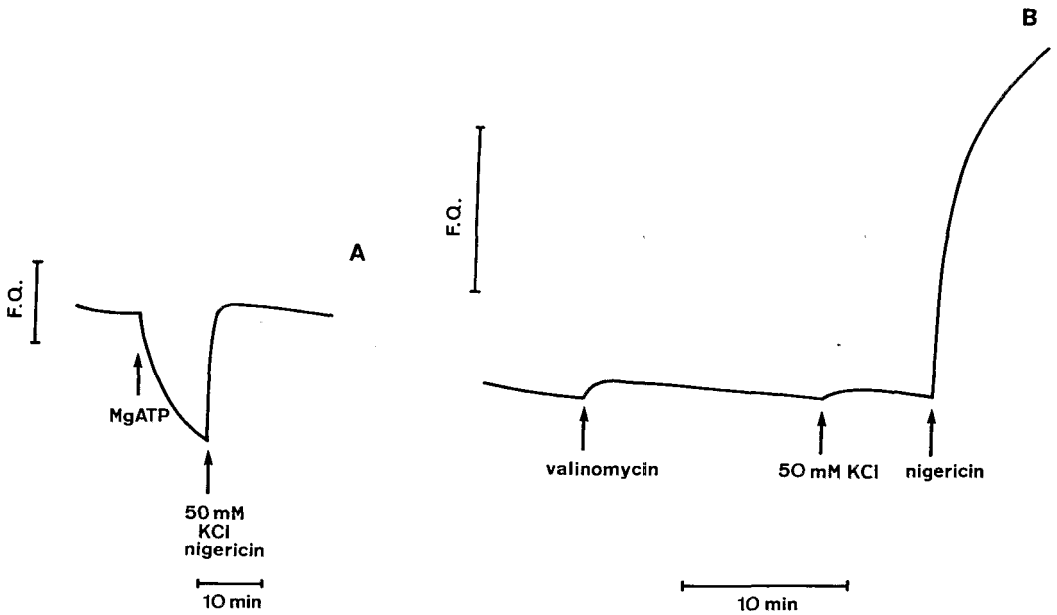

Figure 4. Effect of nigericin and valinomycin on Hevea tonoplast ATPase activity.

Experimental conditions were identical to those in Figure 1. Additions were as follows : 10 µl KCl (1M), 10 µl nigericin (5 mg x ml^{-1}) and 10 µl valinomycin (5 mg x ml^{-1}).

or efflux of DMO (6 - 9). However, as the use of methylamine as ΔpH probe could be criticable, we have used here the method consisting on monitoring the quenching of fluorescence of an acridine derivative (ACMA) in relation with the transmembrane ΔpH changes. By this way, we have confirmed the preexisting data obtained by other methodological means (7,9).

Moreover, we insist on the lack of effect of two typical plasmalemma ATPase inhibitors, i.e. vanadate and diethystilboestrol, on the Hevea tonoplast ATPase. Such a result could be considered as significant to oppose two membrane ATPases which act as proton pump and which are located on membranes which limit the cytoplasm space : the plasmalemma by the external side, and the tonoplast by the internal side. Consequently, these two ATPases could be considered as proton efflux pumps, the flux being originated from the cytoplasm and finishing either in the external medium (through plasmalemma proton

pump) or in the vacuolar medium (through the tonoplast proton pump).

From the experiments with FCCP, we should evoke here again the probable contribution of a Donnan effect for a large part in the preexisting Hevea tonoplast ΔpH (8,9). Moreover, the effects of ionophore 1799 and nigericin in the presence of KCl are clearly in accordance with the hypothesis of a surimposed ΔpH induced by an ATP-dependent proton pump.

The occurrence of such an enzyme at the tonoplast level in higher plants is very important and, at least, in Hevea latex, its activity constitutes the driving force for soluble transport which are often trapped by vacuolar indiffusible molecules once into the internal tonoplast compartment (6,20).

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