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Solubilization and Reconstitution of the $Mg^{2+}/2H^+$ Antiporter of the Lutoid Tonoplast from *Hevea brasiliensis* Latex

Zakia Amalou[1], Rémy Gibrat*, Pierre Trouslot, and Jean d'Auzac

Biochimie et Physiologie Végétales, Ecole Nationale Supérieure Agronomique de Montpellier/Institut National de la Recherche Agronomique/Centre National de la Recherche Scientifique (Unité de Recherche Associée 573), F-34060 Montpellier Cedex 1, France (Z.A., R.G.); Laboratoire d'Agrophysiologie, Centre de Coopération Internationale de Recherche Agronomique pour le Développement-Culture Perennes, F-34032 Montpellier Cedex 1 BP 5035, France (Z.A.); Laboratoire de Biotechnologie, Physiologie et Métabolisme Cellulaires, Institut Français de Recherche Scientifique pour le Développement en Coopération, 911 Av Agropolis Montpellier BP 5045, France (P.T.); and Laboratoire de Biotechnologie et de Physiologie Végétale Appliquée, Université Montpellier 2, F-34095 Montpellier Cedex 5, France (J.d'A.)

The $Mg^{2+}/2H^+$ antiporter recently described on lutoid membrane (Z. Amalou, R. Gibrat, C. Brugidou, P. Trouslot, J. d'Auzac [1992] Plant Physiol 100: 255–260) was solubilized by octylglucoside and reconstituted into soybean liposomes using the detergent dilution method. Magnesium efflux or influx experiments were used to generate a H^+ influx or efflux, respectively, monitored with the fluorescent probe 9-amino-6-chloro-2-methoxyacridine. Both experiments gave saturable H^+ fluxes as a function of internal or external Mg^{2+} concentrations with similar kinetic parameters K_m and V_{max}. The K_m value for Mg^{2+} (about 2 mM) was identical to that previously found in lyophilized-resuspended lutoid (reference therein), whereas the V_{max} value was 14-fold higher. Since only 10% of the initial proteins were recovered in proteoliposomes, and electrophoretic patterns of the two kinds of vesicles differed significantly, it was inferred that the increase in V_{max} was due essentially to an enrichment of the protein antiporter in the reconstituted fraction, owing to a selective effect of octylglucoside at both solubilization and reconstitution steps. None of the various divalent cations used could dissipate the pH gradient of control liposomes of soybean lipids, unless the divalent/H^+ exchanger A23187 was added, whereas a rapid dissipation of the pH gradient was observed with reconstituted proteoliposomes from lutoid proteins, with the cation selectivity sequence $Zn^{2+} > Cd^{2+} > Mg^{2+}$ in the millimolar concentration range. The divalent ions Ca^{2+}, Ba^{2+}, and Mn^{2+} were incapable of generating a H^+ efflux in reconstituted proteoliposomes, whereas both Mg^{2+}/H^+ and Ca^{2+}/H^+ exchanges were observed in lyophilized-resuspended lutoids. Therefore, the lutoid membrane seems to contain separate Mg^{2+}/H^+ and Ca^{2+}/H^+ transport systems, the latter being eliminated during the solubilization/reconstitution of lutoid membrane proteins.

Magnesium is the second most abundant cation after potassium in the cytoplasm of plant and animal cells. This divalent cation is a necessary cofactor or cosubstrate for numerous enzymes and is involved in molecular or macromolecular structures (e.g. Chl, ribosomes). Magnesium is crucial to many cellular functions, including solute compartmentation, DNA replication and transcription, protein synthesis, glycolysis, oxidative phosphorylation, and photosynthesis (Marschner, 1986). In spite of this important physiological role, data on Mg^{2+} membrane transport systems and compartmentation in plant cells are scarce.

We have previously described a Mg^{2+}/H^+ exchange at the lutoid membrane from *Hevea brasiliensis* latex using fluorescent probes in vitro (Amalou et al., 1992). Latex is the fluid cytoplasm of the laticiferous vessels containing lutoids, a specialized vacuo-lysosomal compartment (d'Auzac et al., 1989). Magnesium is accumulated 10-fold in lutoids compared to the latex cytosol (d'Auzac et al., 1989) and is directly involved in the biosynthesis of *cis*-polyisoprene molecules (Jacob et al., 1989; Kekwick, 1989).

The Mg^{2+}/H^+ exchange was saturable with similar K_m for Mg^{2+} in both Mg^{2+} influx and efflux experiments and was inhibited by amiloride and imipramine. The K_m for Mg^{2+} (2.5 mM) and the inhibitor concentrations for half-maximal inhibition (0.3 and 0.12 mM for amiloride and imipramine, respectively) were close to those determined for the electroneutral $Mg^{2+}/2Na^+$ antiporter in animal cells (Günther et al., 1990). When the membrane potential of lutoid vesicles was clamped to zero with K^+ and valinomycin, the K_m and V_{max} parameters did not change. On the other hand, Mg^{2+} gradients did not generate any detectable membrane potentials, whereas imposition of K^+ gradients in the presence of valinomycin generated high membrane potentials. It was concluded that the lutoid membrane contains an electroneutral $Mg^{2+}/2H^+$ antiporter.

The present paper describes the reconstitution in soybean lipids of a solubilized protein fraction enriched in the lutoid $Mg^{2+}/2H^+$ antiporter and gives the selectivity sequence for divalent cations.

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* Corresponding author; fax 33–67–52–57–37.

Abbreviations: ACMA, 9-amino-6-chloro-2 methoxyacridine; BTP, bis-tris-propane; CMC, critical micellar concentration.

MATERIALS AND METHODS

Plant Material

Latex was obtained from rubber trees (*Hevea brasiliensis*) at the experimental plantation of Institut de Recherches sur le Caoutchouc at Bimbresso, Abidjan, Côte d'Ivoire. Lutoids were isolated from the latex by centrifugation (40,000*g*, 60 min, Beckman J 20 rotor, r_{max} at 4°C), washed five times in buffer containing 300 mM mannitol, 50 mM Hepes-Tris (pH 7.5), and then lyophilized (Marin et al., 1981). Resuspension of lyophilized lutoids with a Potter homogenizer gives tight vesicles, with functional H^+-ATPase and $Mg^{2+}/2H^+$ antiporter (Amalou et al., 1992).

Solubilization of the Lutoid Tonoplast Proteins

All procedures were conducted at 4°C. Lyophilized lutoids were resuspended in buffer A containing 5 mM Hepes-BTP (pH 8.0) and 3 mM $MgSO_4$, assayed for the protein content, and adjusted to a final concentration of 10 mg/mL. The suspension was centrifuged (40,000*g*, 30 min, Beckman J 20 rotor, r_{max}) and the pellet, resuspended in 2.5 mL, was solubilized at an optimum octylglucoside concentration of 60 mM in buffer B containing 5 mM Hepes-BTP (pH 8.0), 3 mM $MgSO_4$, 100 mM KCl, and 20% (v/v) glycerol. This mixture was incubated on ice and stirred for 30 min. A clear supernatant was obtained after centrifugation (145,000*g*, 1 h, Beckmann Ti 50 rotor, r_{max}) and assayed for solubilized proteins. A thin, rubber film at the top of the tube was discarded before collecting the solubilized proteins in the supernatant.

Reconstitution of the $Mg^{2+}/2H^+$ Antiporter

Liposomes from soybean lipids (L-α-phosphatidylcholine, type II-S, Sigma) were prepared as follows: 40 mg of lipids were dispersed by vigorous mixing on a vortex mixer in the presence of glass beads in 1 mL of buffer B for 15 min under argon. Afterward, the suspension of multilayer vesicles was sonicated for 15 min in a Bransonic bath sonicator until clarification. Sonicated liposomes were solubilized by octylglucoside. Solubilization was monitored by measuring the decrease of the turbidity of the liposome suspension as a function of detergent concentration (not shown). Liposomes were fully solubilized at an effective ratio value [Re = (detergent − CMC)/(phospholipids)] of 3.0 where concentrations are expressed in mM. The value used for the CMC of octylglucoside was 25 mM: this was confirmed using the fluorescent anilino-naphthalene-sulfonate probe, the quantum yield of which strongly increased upon its binding on detergent micelles (not shown). The phospholipid/detergent mixture was added to the solubilized proteins at an optimum phospholipid:protein ratio of 15 and placed on ice for 30 min. Due to the high CMC of octylglucoside, solubilized membrane proteins were reconstituted using the detergent dilution method (Racker et al., 1979), as already achieved for a variety of divalent ion antiporters (Miyamoto and Racker, 1980; Nakamura et al., 1986; Schumaker and Sze, 1990). The protein/phospholipid/detergent mixture (2.5 mL) was slowly injected into 125 mL of buffer B without glycerol and incubated and stirred for 30 min at 25°C. The clear lipid-protein mixture became cloudy and was centrifuged (145,000*g*, 1 h, Beckman Ti 70 rotor, r_{max}), resuspended in 0.25 mL of buffer B, and stored in liquid nitrogen. Proteoliposomes were diluted an additional 200-fold in the assay cuvette to measure the initial rate of ACMA quenching as described below.

Transport Experiments

In Mg^{2+} efflux experiments, membrane vesicles loaded with the indicated $MgSO_4$ concentration in 5 mM Hepes-BTP (pH 8.0) were diluted (5–25 μg/mL protein) in the assay medium (2 mL) containing the same $MgSO_4$ concentration, 25 mM Hepes-BTP (pH 8.0), and 1 μM ACMA. A quasi-infinite outward diffusion gradient of Mg^{2+} was generated by addition to the outside of EDTA-BTP (pH 8.0) in excess of the Mg^{2+} concentration. The $Mg^{2+}/2H^+$ antiporter used this gradient to generate a Mg^{2+} efflux coupled to a H^+ influx, which was monitored at 30°C using the permeant fluorescent pH probe ACMA. Fluorescence quenching of ACMA was measured at 415/485 nm excitation/emission wavelengths with an SLM 8000C spectrofluorometer. The H^+ influx, measured as the initial rate of ACMA quenching (V_H+), was linear with protein concentration and was expressed in specific units (% quenching min^{-1} μg^{-1} protein).

In divalent influx experiments, membrane vesicles were loaded with 5 mM NH_4SO_4, 0.5 mM EDTA-BTP (pH 8.0), 1 mM Hepes-BTP (pH 8.0) and were diluted 200-fold (5–25 μg/mL protein) in the assay cuvette (2 mL) containing 5 mM Hepes-BTP (pH 8.0) and 1 μM ACMA. The NH_4^+ dilution resulted in a quasi-instantaneous acid loading of the vesicles, due to the outward diffusion of NH_3 (Schumaker and Sze, 1990). The corresponding quenching of the ACMA probe was high and stable. Thereafter, quasi-infinite inward gradients were imposed by adding Mg^{2+}, or the indicated divalent cations, to the outside; the $Mg^{2+}/2H^+$ antiporter used this gradient to generate a divalent influx coupled to a H^+ efflux, estimated by the initial rate of dissipation of the ACMA quenching (also designated V_H+).

The effect of amiloride and imipramine was studied in Mg^{2+} efflux experiments by measuring V_H+ after incubating proteoliposomes for 10 min at the indicated inhibitor concentrations in the assay medium. Amiloride interfered with the fluorescence of permeant pH dye (Sabolic and Burchkardh, 1983). Correction for this interference was performed by adding amiloride to the ACMA-containing assay cuvette prior to adding the vesicles and changing the signal amplification to recover the initial fluorescence intensity (Blumwald and Poole, 1985).

SDS-PAGE of Lutoid Membrane Proteins

SDS-PAGE was performed in a Bio-Rad gel apparatus by the method of Laemmli (1973). Electrophoresis in the 1.5-mm-thick slab gels was carried out on a 10% (w/v) acrylamide gel with a stacking gel of 4% (w/v) acrylamide. Samples were diluted 2-fold in buffer containing 62 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 1% 2-mercaptoethanol, and 0.001% (w/v) bromphenol blue and incubated for 5 min at 100°C prior to electrophoresis. The gels were silver stained (Oakley et al., 1980) and the

molecular mass of proteins was estimated by comparison to standard proteins.

Acid Phosphatase and α-Mannosidase Assays

Acid phosphatase (EC 3.1.3.2) and α-mannosidase (EC 3.2.1.24) were measured as described elsewhere (d'Auzac, 1981). Samples were incubated at 30°C for 30 min in buffer containing 0.1 M acetate (pH 5.0), 0.1% (v/v) Triton X-100, and 5 mM *p*-nitrophenyl-phosphate or *p*-nitrophenyl-α-mannoside respectively. The activities were measured by the release of *p*-nitrophenol.

Protein Assay

Proteins were estimated by the method of Schaffner and Weissmann (1973).

Chemicals

ACMA was purchased from Molecular Probes (Eugene, OR), and all other chemicals were from Sigma.

RESULTS

Only one-half of the initial protein content of lyophilized and resuspended lutoid vesicles was recovered in the pellet after a single wash in the buffer without detergent (Table I). Approximately 80% of the activity of two soluble enzymic markers of the vacuolar compartment (Matile, 1978; d'Auzac, 1981), acid phosphatase and α-mannosidase, were also lost after this wash. This suggests that the compartmentation of soluble proteins in native lutoids, which was maintained after five washes in an isoosmotic buffer performed at the experimental plantation of Institut de Recherches sur le Caoutchouc was lost when lutoids were resuspended after lyophilization. The maximum specific rate of the Mg^{2+}/H^+ exchange, measured in Mg^{2+} efflux experiments as described in "Materials and Methods," increased 2-fold, in agreement with the above-mentioned loss of soluble proteins (Fig. 1, trace b; Table I).

Almost 70% of the remaining membrane proteins in the washed pellet could be solubilized by octylglucoside under the optimum conditions indicated in "Materials and Methods," and only 30% of the solubilized proteins could be reconstituted after the detergent dilution step. Silver staining of the SDS-polyacrylamide gel revealed that several bands were highly enriched after the reconstitution step (Fig. 2).

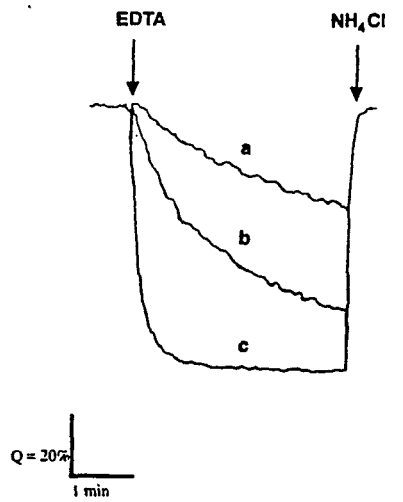

Figure 1. H^+/Mg^{2+} exchange activity in the different vesicle fractions. Mg^{2+} efflux was initiated by addition of EDTA (4 mM) to the exterior of vesicles (2.5 mg/mL protein) equilibrated with a buffer containing 3 mM $MgSO_4$. Traces are the quenching of ACMA fluorescence measured as described in "Materials and Methods." Trace a, Lyophilized-resuspended lutoid vesicles; trace b, washed lutoid vesicles; trace c, reconstituted proteoliposomes (see text). Addition of 10 mM NH_4Cl abolished the pH gradient.

The kinetic parameters (K_m for Mg^{2+} and V_{max}) of the Mg^{2+}/H^+ exchange in reconstituted vesicles were almost the same from both Mg^{2+} efflux or influx experiments (2 mM and 180% quenching min^{-1} μg^{-1} protein, respectively, Fig. 3, A and B). The V_{max} value was 14-fold higher than that obtained with lyophilized-resuspended lutoids (Amalou et al., 1992; Fig. 1; Table I). As previously shown, the Mg^{2+}/H^+ exchange was inhibited by amiloride and imipramine and the half-inhibiting concentrations were 0.16 and 0.070 mM, respectively (Fig. 4).

Selectivity of the divalent/proton exchange in reconstituted vesicles was determined by measuring the dissipation of preformed H^+ gradients upon imposition of quasi-infinite inward gradients of divalents (according to the acid-loading

Table I. *Solubilization and reconstitution of the $Mg^{2+}/2H^+$ antiporter:Mg^{2+}-dependent acidification rate and activities of soluble enzymes*

The origin of the different fractions and the assay of the different activities are described in "Materials and Methods."

Fraction	Proteins	Soluble Activities		Acidification Rate
		Acid Phosphatase	α-Mannosidase	
	mg	$\mu mol\ h^{-1}\ mg^{-1}$ protein		% min^{-1} μg^{-1} protein
Lyophilized lutoids	10.0	7.2	0.60	7
Supernatant	5.0	6.0	0.50	nd
Washed pellet	5.0	1.4	0.12	15
Solubilized	3.5	nd[a]	nd	nd
Reconstituted[b]	1.0	nd	nd	100

[a] nd, Not detectable. [b] At lipid/protein ratio of 15.

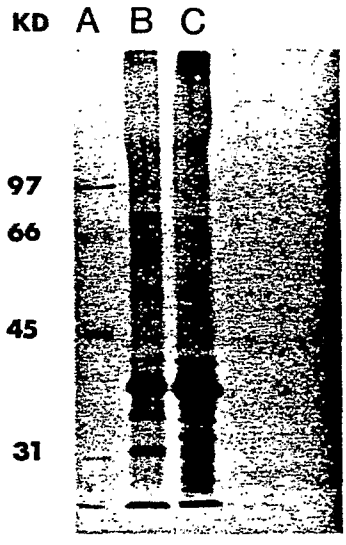

Figure 2. SDS-polyacrylamide gel electrophoretic pattern of washed lutoids and reconstituted proteoliposomes. Proteins were run in 10% polyacrylamide gels and subsequently silver stained. The bars indicate the positions of the following mol wt protein standards: phosphorylase *b* (97,000); BSA (66,000); ovalbumin (45,000); bovine carbonic anhydrase (31,000). Gel lanes show proteins from protein standards (A), washed lutoids (B), and reconstituted proteoliposomes (C).

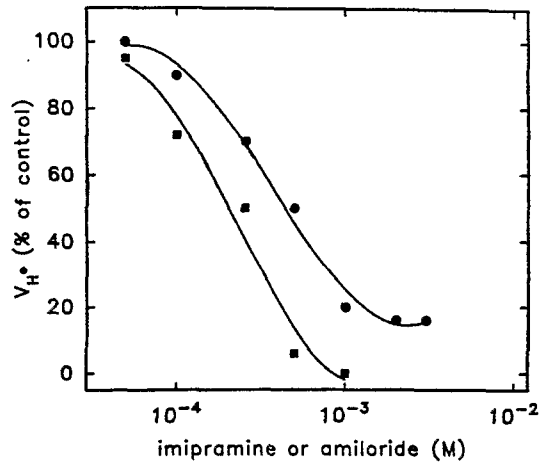

Figure 4. Effects of amiloride (●) and imipramine (■) on H^+/Mg^{2+} exchange by reconstituted proteoliposomes. Proteoliposomes were reconstituted as described in "Materials and Methods." The antiporter activity was assayed as in Figure 1, after incubation at 20°C for 10 min in the presence of the indicated amiloride and imipramine concentrations. The V_H^+ values are expressed as percent of control (V_H^+ = 180% min^{-1} mg^{-1} protein).

protocol described in "Materials and Methods"). A control experiment on reconstituted liposomes indicated that none of the divalents used could dissipate the preformed H^+ gradient (Fig. 5A). When reconstituted liposomes contained the ionophore A23187, a rapid dissipation was observed after addition of 0.1 mM divalents with the selectivity sequence $Cd^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. None of the divalents used, however, were capable of dissipating the H^+ gradient across proteoliposomes when added at 0.1 mM (Fig. 5B). At 1 mM, a rapid dissipation of the H^+ gradient was observed with the selectivity sequence $Zn^{2+} > Cd^{2+} > Mg^{2+}$, whereas Ca^{2+}, Ba^{2+}, or Mn^{2+} were inefficient. This strongly differed from the results obtained with lyophilized-resuspended lutoid vesicles in divalent cation efflux experiments (Fig. 6). In this case, a H^+/Ca^{2+} exchange activity was observed together with a H^+/Mg^{2+} activity, with V_{max} values of 6.2 and 2.1% quenching min^{-1} μg^{-1} protein, respectively, from experiments

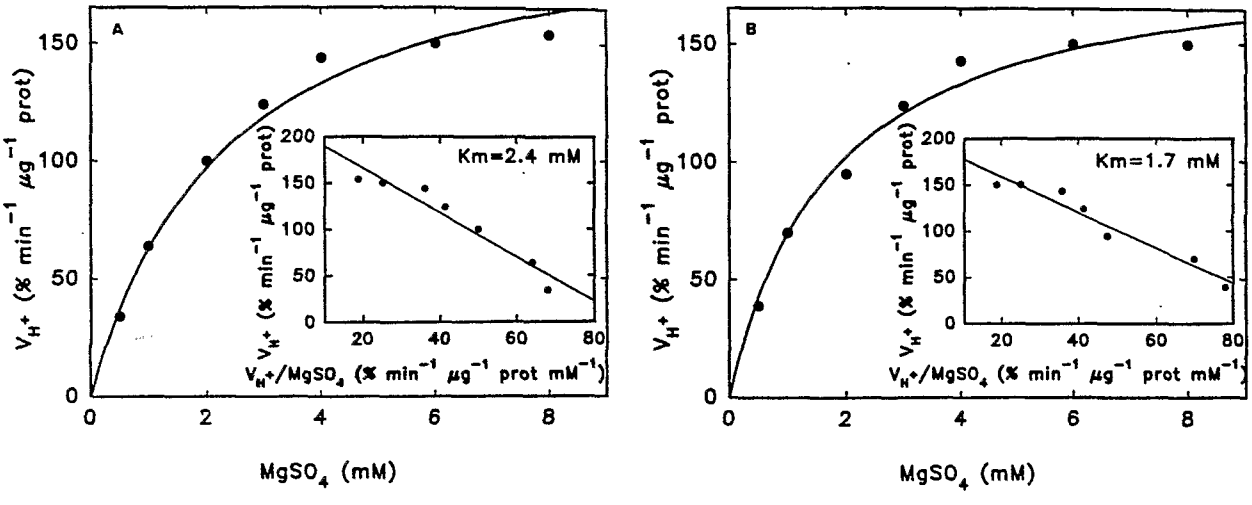

Figure 3. H^+/Mg^{2+} exchange by reconstituted proteoliposomes as a function of Mg^{2+} concentration. Proteoliposomes were reconstituted and the initial rate of ACMA fluorescence quenching (V_{H^+}) was determined as described in "Materials and Methods." A, The acidification reaction resulted from infinite outward Mg^{2+} gradients following EDTA addition to the outside of proteoliposomes loaded with increasing Mg^{2+} concentrations. B, Effect of Mg^{2+} concentration on rate of recovery of fluorescence after an imposed pH difference by acid-loading method. Insets, Eadie-Hofstee plots.

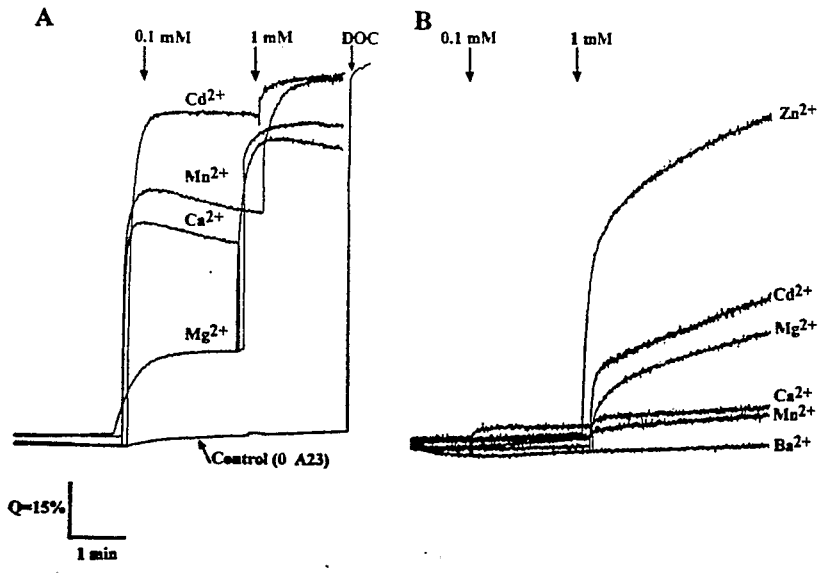

Figure 5. Selectivity of the proton/divalent cation exchange by liposomes and reconstituted proteoliposomes. Traces show recovery of ACMA fluorescence, corresponding to dissipation of the pH gradient, in acid-loaded liposomes or proteoliposomes (see "Materials and Methods"). Arrows indicate the points at which aliquots of divalent cations (0.1 or 1 mM) or deoxycholate (DOC, 0.05% [v/v] final) were added. A, Liposomes; the addition of the different divalent cations did not dissipate the pH gradient when A23187 was absent (control), whereas rapid dissipations were obtained in its presence (0.5 μM). B, Proteoliposomes.

involving saturating concentrations of divalent cations inside the vesicles and infinite outward gradients.

DISCUSSION

None of the divalent cations used in this study could be exchanged with H^+ when added to the outside of acid-loaded liposomes of soybean lipids, even in the millimolar range (Fig. 5A). This confirms that nonfacilitated (electrophoretic) divalent/H^+ exchange does not occur at the lipidic bilayer used in protein reconstitution, as previously shown in Mg^{2+} efflux experiments (Amalou et al., 1992). In the presence of the ionophore A23187, a rapid exchange was observed with the selectivity sequence $Cd^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$ known for this ionophore (Pressman, 1976). This indicates that the experimental protocol was suitable to detect a facilitated divalent/H^+ exchange at a relatively low concentration of divalents (0.1 mM), and allowed the reliability of the selectivity sequence. Therefore, the inefficiency of divalent cations at this concentration in dissipating the preformed H^+ gradient across reconstituted proteoliposomes confirms that the divalent/H^+ antiporter of the lutoid tonoplast displays a low affinity. In the millimolar range, a rapid exchange was observed with the selectivity sequence $Zn^{2+} > Cd^{2+} > Mg^{2+}$ (Fig. 5B). The Mg^{2+}/H^+ exchange of proteoliposomes was saturable (K_m = 2 mM, V_{max} = 180% quenching min^{-1} μg^{-1} protein; Fig. 3) and inhibitable by amiloride and imipramine (Fig. 4).

Since the divalent/H^+ exchange is saturable in the millimolar range and inhibitable, it can be attributed to a transport protein. Since Mg^{2+} is the only transported divalent present in the 1-mM range in the cytoplasm of plant (Yazaki et al., 1988) and animal cells (Heinonen and Akermann, 1987; Ishii and Lehrer, 1989), this protein must be physiologically involved in Mg^{2+} compartmentation. We have previously shown on lyophilized-resuspended lutoids that Mg^{2+} influx did not generate a membrane potential (using the membrane potential probe oxonol VI), and that K^+-valinomycin did not affect the Mg^{2+}/H^+ exchange (Amalou et al., 1992). Therefore, it was concluded that the lutoid membrane contains an electroneutral $Mg^{2+}/2H^+$ antiporter.

The half-inhibiting concentrations (Fig. 4) of amiloride (0.16 mM) and imipramine (0.070 mM) and K_m for Mg^{2+} (2 mM; Fig. 3) were consistent with those previously obtained in lyophilized-resuspended lutoids (0.3 and 0.12 mM for the inhibitors and 2.6 mM for Mg^{2+}, respectively; Amalou et al., 1992). Thus, the solubilization-reconstitution protocol used

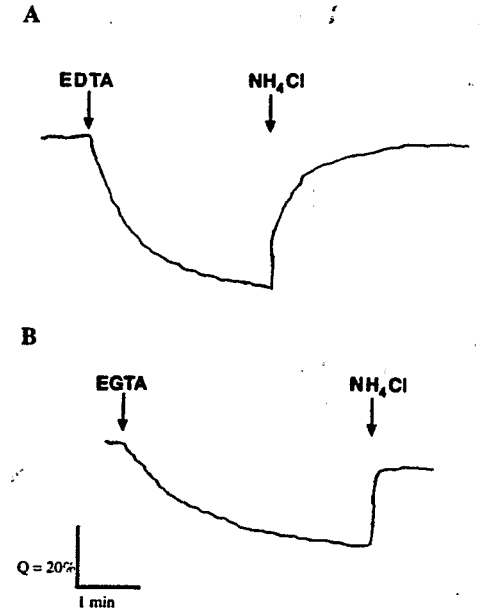

Figure 6. H^+/Mg^{2+} and H^+/Ca^{2+} exchange activities by lyophilized-resuspended lutoid vesicles. The acidification reaction resulted from infinite outward Mg^{2+} (A) or Ca^{2+} (B) gradients following EDTA (6 mM) or EGTA (0.6 mM) addition to the exterior of vesicles (12.5 mg/mL protein), equilibrated with a buffer containing 5 mM $MgSO_4$ or 0.5 mM $CaSO_4$ (corresponding to the maximum H^+ influx in each situation). Addition of 10 mM NH_4Cl abolished the pH gradient.

in this study does not significantly modify the intrinsic transport properties of the antiporter.

The V_{max} parameter in the proteoliposomes increased 14-fold compared to that for lyophilized-resuspended lutoids (Table I). Several factors must be considered to explain such an augmentation. First, the ACMA response will depend on the internal buffering of the vesicles. In the present study, the different kinds of vesicles contained the same buffer. Second, the increase of the V_{max} parameter could be due to an increase of the membrane tightness to H^+. This hypothesis is unlikely because the pH gradient of acid-loaded vesicles by NH_4^+ dilution was very stable in both liposomes and proteoliposomes (Fig. 5), as well as in lyophilized-resuspended lutoids (Amalou et al., 1992); in each case the pH gradient dissipation was totally dependent on the facilitated Mg^{2+} influx.

Third, a variation in the statistical orientation of antiporter molecules after protein reconstitution could also be responsible for variation in the V_{max} parameter if the antiporter displays a large asymmetry of the kinetic parameters for influx and efflux. The similarity of the two kinetic parameters K_m and V_{max} previously observed in Mg^{2+} efflux and influx experiments on lyophilized-resuspended lutoids (Amalou et al., 1992) and on reconstituted vesicles (this work) do not imply an actual symmetry of the molecular kinetic parameters of the antiporter for the following reasons. The starting preparations of lyophilized-resuspended lutoids appear to contain approximately half inside-out and half right-side-out vesicles, as estimated from the latency of the hydrolytic activity of the H^+-ATPase (data not shown). On the other hand, reconstitution using the detergent dilution method, as in the present work, leads generally to a scrambling of the molecular orientation of membrane proteins (Helenius et al., 1981; Eytan, 1982). Thus, even if the molecular kinetic parameters of the antiporter should be highly asymmetric, similar statistical kinetic parameters K_m and V_{max} can be determined from both Mg^{2+} efflux and influx experiments performed in this study due to a scrambling of the membrane vesicle orientation in lyophilized-resuspended vesicles, or the expected scrambling of antiporter molecules in the reconstituted membrane. The important point is that such modifications of orientation do not explain the increase of the V_{max} parameter, since antiporter molecules are expected to be half-oriented right-side-out in all experimental situations used in this study.

Another important parameter involved in the ACMA response is the surface:volume ratio of the vesicles. Larger vesicles will exhibit larger fluorescence quenches, but smaller vesicles will exhibit higher initial rates (Bennett and Spanswick, 1982). The size of resuspended lyophilized lutoids is heterogeneous but larger than those of reconstituted vesicles, since a high proportion of lutoid vesicles are visible under a photonic microscope. This could explain the observed increase in the initial rate of transport after reconstitution, which should be proportional in theory to the decrease of the vesicle radius. Nevertheless, this effect is expected to be significantly counterbalanced by the decrease of the density of membrane proteins at the surface of the reconstituted vesicles (membrane proteins were 15-fold diluted in soybean lipids).

Finally, a significant proportion of the increase of the V_{max} parameter in reconstituted vesicles seems likely to be due to an actual enrichment in antiporter molecules. The total activity (i.e. the specific activity × protein amount) in the initial fraction (lyophilized-resuspended lutoids) was recovered in the reconstituted fraction (Table I), which contained only 10% of the initial proteins. Indeed, the different protein fractions exhibited different electrophoretic patterns, indicating that several polypeptides were enriched (Fig. 2). It has to be pointed out that half of the protein content in lyophilized-resuspended lutoids were simultaneously lost with soluble markers of the vacuole after a single washing, and that only 20% of the proteins in the washed pellet were solubilized and reconstituted using octylglucoside and the present protocol. Selective effects of detergents have been widely used in a first step of membrane protein purification. For instance, Triton X-100 washing in the presence or absence of chaotropic agent (KI or KBr) has been shown to cause significant enrichments in plasma membrane H^+-ATPase (Grouzis et al., 1987; Sandstrom and Cleland, 1989).

Due to the physiological role of Mg^{2+}, it is important to determine if such a system exists in membranes other than the specialized lutoid membrane. A Mg^{2+}/H^+ exchange was recently shown in native tonoplast vesicles isolated from maize roots (Pfeiffer and Hager, 1993). The divalent/H^+ antiport activity and affinity were higher for Ca^{2+} than for Mg^{2+} in maize root tonoplast. Since the observed affinity for Ca^{2+} was much lower than its normal cytoplasmic concentration, it was inferred that the divalent/H^+ antiporter of maize tonoplast was physiologically involved in the transport of Mg^{2+}, and not of Ca^{2+}. Another hypothesis could be raised from the present work: the tonoplast membrane could contain two distinct divalent/H^+ antiporters, discriminating Ca^{2+} and Mg^{2+}, which are involved in totally different physiological processes. The Ca^{2+}/H^+ exchange already shown in the native lutoid membrane (Chrestin et al., 1984) was recovered in lyophilized-resuspended lutoids (Fig. 6) but it was totally absent in reconstituted proteoliposomes (Fig. 5B). This suggests that the Ca^{2+}/H^+ antiporter has been eliminated during the solubilization-reconstitution step (only 10% of the initial protein content was recovered in these vesicles). In animal cells, it has been previously proposed that the same transporter may be responsible for both Mg^{2+} and Ca^{2+} transport (Mullins et al., 1977), but it appears now that Mg^{2+} needs its own, separate transport system (see Flatman, 1991, for a review).

Identification of the polypeptide(s) involved in the $Mg^{2+}/2H^+$ antiport at the lutoid membrane will necessitate further steps of protein purification. The ability to solubilize and reconstitute the $Mg^{2+}/2H^+$ antiporter while maintaining its transport properties is important in such a strategy.

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LITERATURE CITED

Amalou Z, Gibrat R, Brugidou C, Trouslot P, d'Auzac J (1992) Evidence for an amiloride-inhibited $Mg^{2+}/2H^+$ antiporter in lutoid (vacuolar) vesicles from latex of *Hevea brasiliensis*. Plant Physiol **100:** 255–260

Bennett AB, Spanswick RM (1982) Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles. Kinetics analysis of Cl^- effects on proton translocating ATPase. J Membr Biol **71:** 95–107

Blumwald E, Poole RJ (1985) Na^+/H^+ antiporter in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol **78:** 163–167

Chrestin H, Gidrol X, Jacob JL, d'Auzac J (1984) Role of the lutoidic tonoplast in control of the cytosolic homeostasis within the laticiferous cells of *Hevea*. Z Pflanzenphysiol **114:** 269–277

d'Auzac J (1981) Une α-mannosidase dans le système vacuolaire végétal: les lutoïdes du latex d'*Hevea brasiliensis*. CR Acad Sci Paris Ser III **292:** 1085–1087

d'Auzac J, Jacob JL, Chrestin H (1989) The composition of latex from *Hevea brasiliensis* as a laticiferous cytoplasm. *In* J d'Auzac, JL Jacob, H Chrestin, eds, Physiology of Rubber-Tree Latex. CRC Press, Boca Raton, FL, pp 59–96

Eytan GD (1982) Use of liposomes for reconstitution of biological functions. Biochim Biophys Acta **694:** 185–202

Flatman PW (1991) Mechanisms of magnesium transport. Annu Rev Physiol **53:** 259–271

Grouzis JP, Gibrat R, Rigaud J, Grignon C (1987) Study of sidedness and tightness to H^+ of corn root plasmalemma vesicles: preparation of a fraction enriched in inside-out vesicles. Biochim Biophys Acta **903:** 449–464

Günther T, Vorman J, Höllriegl V (1990) Characterization of Na^+-dependent Mg^{2+} efflux from Mg^{2+}-loaded rat erythrocytes. Biochim Biophys Acta **1023:** 455–461

Heinonen E, Akermann KEO (1987) Intracellular free magnesium in synaptosomes measured with entrapped eriochrome blue. Biochim Biophys Acta **898:** 331–337

Helenius A, Sarvas M, Simons K (1981) Asymmetric and symmetric membrane reconstitution by detergent elimination. Eur J Biochem **116:** 27–35

Ishii Y, Lehrer SS (1989) Mg^{2+} paracrystal formation of tropomyosin as a condensation phenomenon. Biophys J **56:** 107–114

Jacob JL, Prévôt JC, Kekwick RGO (1989) General metabolism of *Hevea brasiliensis* latex (with the exception of isoprenic anabolism). *In* J d'Auzac, JL Jacob, H Chrestin, eds, Physiology of Rubber-Tree Latex. CRC Press, Boca Raton, FL, pp 101–145

Kekwick RGO (1989) The formation of isoprenoids. *In* J d'Auzac, JL Jacob, H Chrestin, eds, Physiology of Rubber-Tree Latex. CRC Press, Boca Raton, FL, pp 145–164

Laemmli UK (1973) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Marin B, Marin-Lanza M, Komor E (1981) The protonmotive potential difference across the vacuo-lysosomal membrane of *Hevea brasiliensis* (rubber tree) and its modification by a membrane-bound adenosine triphosphatase. Biochem J **198:** 365–372

Marschner H (1986) Mineral Nutrition of Higher Plants. Academic Press, Orlando, FL, pp 237–238

Matile P (1978) Biochemistry and function of vacuoles. Annu Rev Plant Physiol **29:** 193–213

Miyamoto H, Racker E (1980) Solubilization and partial purification of the Ca^{2+}/Na^+ antiporter from the plasma membrane of bovine heart. J Biol Chem **255:** 2656–2658

Mullins LJ, Brinley FJ Jr, Spangler SG, Abercombie RF (1977) Magnesium efflux in dialyzed squid axons. J Gen Physiol **69:** 389–400

Nakamura T, Hsu C, Rosen BP (1986) Cation/proton antiport systems in *Escherichia coli*. J Biol Chem **261:** 678–683

Oakley RB, Kirsch RD, Morris RN (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem **105:** 361–363

Pfeiffer W, Hager A (1993) A Ca^{2+}-ATPase and a Mg^{2+}/H^+-antiporter are present on tonoplast membranes from roots of *Zea mays* L. Planta **191:** 377–385

Pressman BC (1976) Biological application of ionophores. Annu Rev Biochem **45:** 501–530

Racker E, Violand B, O'Neal S, Alfonzo M, Telford J (1979) Reconstitution, a way of biochemical research: some new approaches to membrane-bound enzymes. Arch Biochem Biophys **198:** 470–477

Sabolic I, Burchkardh G (1983) Apparent inhibition of Na^+/H^+ exchange by amiloride and harmaline in acridine orange studies. Biochim Biophys Acta **731:** 354–360

Sandstrom RP, Cleland R (1989) Selective delipidation of the plasma membrane by surfactants. Plant Physiol **90:** 1524–1531

Schaffner W, Weissmann C (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal Biochem **56:** 502–514

Schumaker KS, Sze H (1990) Solubilization and reconstitution of the oat root vacuolar H^+/Ca^{2+} exchanger. Plant Physiol **92:** 340–345

Yazaki Y, Asukagawa N, Ishikaway Y, Eiji O, Sakata M (1988) Estimation of cytoplasmic free Mg^{2+} levels and phosphorylation potentials in mung bean root tips by in vivo ^{31}P-NMR spectroscopy. Plant Cell Physiol **29:** 919–924