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Evidence for an Amiloride-Inhibited $Mg^{2+}/2H^+$ Antiporter in Lutoid (Vacuolar) Vesicles from Latex of *Hevea brasiliensis*[1]

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ABSTRACT

Lutoids represent a lysosomal microvacuolar compartment of rubber-tree (*Hevea brasiliensis*) latex. We observed acidification of isolated vesicles after imposing an outward Mg^{2+} diffusion gradient and dissipation of a preformed pH gradient in the presence of exogenous Mg^{2+}. These results suggest the presence of a Mg^{2+}/H^+ antiporter. The maximum Mg^{2+}/H^+ exchange rate was observed at pH 8.5. The K_m values for Mg^{2+} (2.6 mM) were identical for both influx and efflux experiments. When membrane potential was clamped at zero with K^+ and valinomycin, the response of the membrane potential probe oxonol VI showed that the Mg^{2+}/H^+ exchange was electroneutral. Mg^{2+}/H^+ exchange was inhibited by amiloride and imipramine. Both the inhibiting concentration range and the K_m for Mg^{2+} are similar to those reported for the $Mg^{2+}/2Na^+$ antiporter in animals cell. These data are consistent with the existence of a $Mg^{2+}/2H^+$ antiporter in a plant tonoplast.

The latex of the rubber tree (*Hevea brasiliensis* Müll.-Arg. Kunth) consists of the fluid cytoplasmic content of the laticiferous system containing lutoids, a specialized lysosomal microvacuolar compartment (7). Mg^{2+} is the most abundant divalent cation in cells. In the rubber tree, Mg^{2+} is directly involved as a cofactor in regulation of carbohydrate metabolism and in isomerization reactions involved in the production of *cis*-polyisoprene molecules (13, 14). Although the accumulation of the free form of Mg^{2+} is not known, total (free plus bound) Mg^{2+} is accumulated approximately 10-fold in lutoids (7). It is essential for many cellular functions, including DNA transcription, protein synthesis, and other metabolic processes that take place in the cytoplasm (15). In spite of its important role, little information is available on the transport and regulation of Mg^{2+} in plant cells (21).

In this paper, we show that an H^+ gradient was created following the imposition of an Mg^{2+} gradient across lutoid membranes. Conversely, a preformed H^+ gradient was reversed by Mg^{2+} addition. In both cases, amiloride inhibited the transport reaction. The absence of a transmembrane electrical potential generated by Mg^{2+}/H^+ exchange supports the conclusion that this process is catalyzed by an electroneutral $Mg^{2+}/2H^+$ antiporter. This is the first indication of the existence of such an antiporter in a plant tonoplast.

MATERIALS AND METHODS

Plant Material

Latex was obtained from rubber tree (*Hevea brasiliensis*) of the Institut de Recherches sur le Caoutchouc plantations at Bimbresso, Abidjan, Côte d'Ivoire. Lutoids were isolated from latex by centrifugation (40,000*g*, 60 min), and washed five times in buffer containing 300 mM mannitol, 50 mM Hepes-Tris at pH 7.5, and then lyophilized (16). Resuspension of lyophilized lutoids with a Potter homogenizer gives tight vesicles with functional (H^+)ATPase (16). In this study, resuspension medium contained 5 mM Hepes-BTP[2] (impermeant buffer) at the indicated pH, 300 mM mannitol, and $MgSO_4$ or $(NH_4)_2SO_4$ at the indicated concentrations. Control experiments were performed with liposomes from soybean lipids; 40 mg of soybean lipids were dispersed by vigorous mixing on a Vortex mixer in the presence of glass beads in 1 mL of the same buffer as the one used for lutoid vesicles, for 15 min under argon. Afterward, the liposome suspension was clarified by sonication for 15 min under argon in a Bransonic sonicator bath.

Fluorescence Measurements

Fluorescence experiments were carried out with an SLM-Aminco SFP 500 spectrofluorometer. Acidification of lutoids was monitored at 30°C using the permeant fluorescent ACMA probe at 415/485 nm excitation/emission wavelengths. Assay medium (2 mL) contained 5 mM Hepes-BTP (pH 8.5 unless otherwise indicated), 1 mg/mL of BSA (fraction V), 300 mM mannitol, and 1 μM ACMA. When the assay medium also contained Mg^{2+} at equilibrium, an H^+ gradient across lutoid vesicles (25 μg/mL of protein) was generated by the addition of a saturating EDTA concentration. A proton gradient could also be generated by diluting 200-fold lutoid vesicles preloaded with Mg^{2+} (as indicated) or 25 mM

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[2] Abbreviations: BTP, bis-tris-propane; ACMA, 9-amino-6-chloro-2-methoxyacridine; V_H^+, initial rate of quenching; F_o, initial fluorescence; ΔF, increase in the fluorescence.

$(NH_4)_2SO_4$. The V_H^+ was linear with the protein concentration (not shown) and can be expressed in specific units (% quenching min^{-1} μg^{-1} proteins). Transmembrane electrical potential of lutoid vesicles was monitored at 18°C from the fluorescence of the permeant anionic dye oxonol VI at 614/646 nm excitation/emission wavelengths (1). The oxonol VI stock solution contained 2.5 mM dye in ethanol. From this stock solution, dye was diluted daily 50-fold in ethanol:water (1:5, v/v). The cuvette was filled with 2 mL of buffer containing 300 mM mannitol, 25 mM BTP-SO_4 (pH 8.5), 0.5 mM K_2SO_4, and 50 nM oxonol VI. An aliquot of the lutoid vesicle suspension (15 μg/mL of protein) prepared in the same buffer without oxonol was added, giving rise to an increase in the oxonol VI F_o. The fluorescence further increased upon the imposition of a K^+ diffusion potential in the presence of valinomycin. As previously observed, amiloride interfered with the fluorescence of the permeant pH dye (18). 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 to the initial fluorescence intensity (4).

Protein Assay

Proteins were estimated by the method of Schaffner and Weissmann (19).

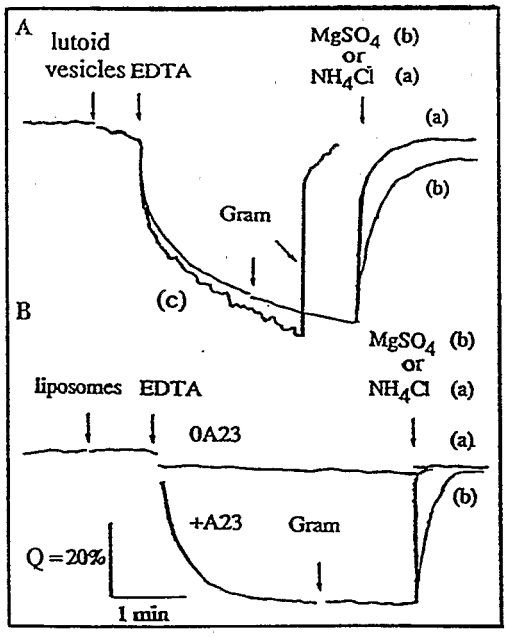

Figure 1. Effect of an outwardly directed Mg^{2+} gradient on the pH difference across vesicles of lutoid from *Hevea* latex and liposomes of soybean lipids. Traces are for the quenching of ACMA fluorescence, measured as described in "Materials and Methods." A, Lutoid vesicles (25 μg/mL of protein) were loaded with 5 mM $MgSO_4$ as described in "Materials and Methods" and were added to 2 mL of buffer containing 5 mM $MgSO_4$, 5 mM Hepes-BTP (pH 8.5), and 1 μM ACMA. Arrows indicate addition of 5.1 mM EDTA, 1 μM gramicidin (Gram), and 50 mM NH_4Cl (trace a) or 10.5 mM $MgSO_4$ (trace b); curve c, gramicidin (Gram) in the presence of 50 mM K_2SO_4. B, Liposomes (200 μg/mL) were loaded and used in parallel with the lutoid vesicles, in the absence or presence of 0.5 μM A23187.

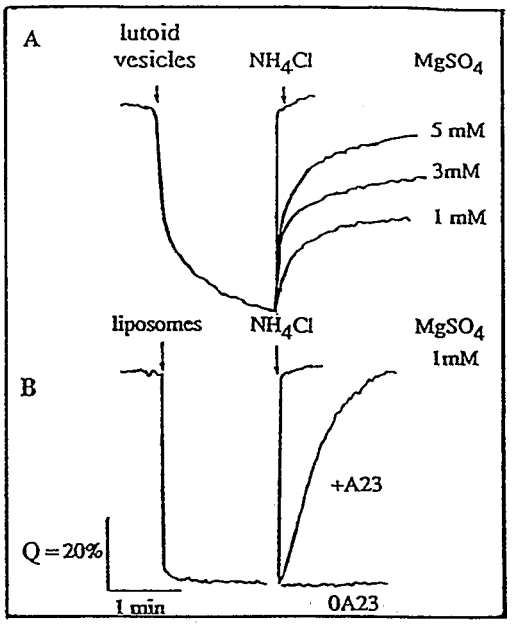

Figure 2. Effect of an inward Mg^{2+} gradient on an imposed pH difference across vesicles of lutoid from *Hevea* latex and liposomes of soybean lipids. The pH difference was imposed by a 200-fold dilution of the vesicles loaded with 25 mM $(NH_4)_2SO_4$ and monitored with the ACMA dye as indicated in "Materials and Methods." Concentrated $MgSO_4$ aliquots were added at the exterior at the indicated concentrations. A, Lutoid vesicles (25 mg/mL of protein); B, liposomes (200 mg/mL) were prepared and assayed as for lutoid vesicles in the absence and presence of 0.5 μM A23187.

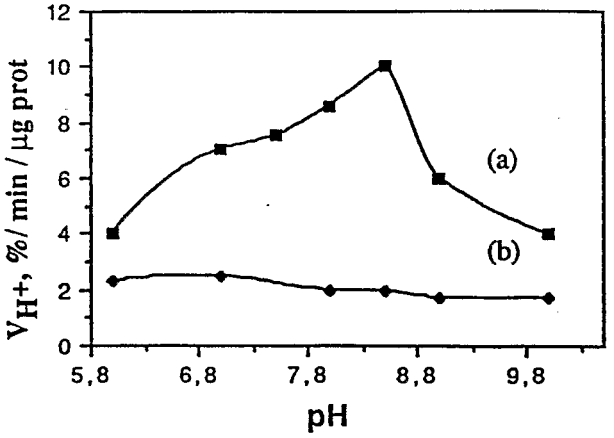

Figure 3. H^+/Mg^{2+} exchange by lutoid vesicles (a) as a function of pH. Lutoid vesicles (25 μg/mL of protein) were loaded with 5 mM Mg^{2+} at the indicated pH and assayed at the same pH. The exchange reaction was started by diluting the vesicles in the same buffer except that Mg^{2+} was omitted. V_H^+ was determined as indicated in "Materials and Methods." Liposomes (b) (200 μg/mL) were loaded and used in the same manner as lutoid vesicles.

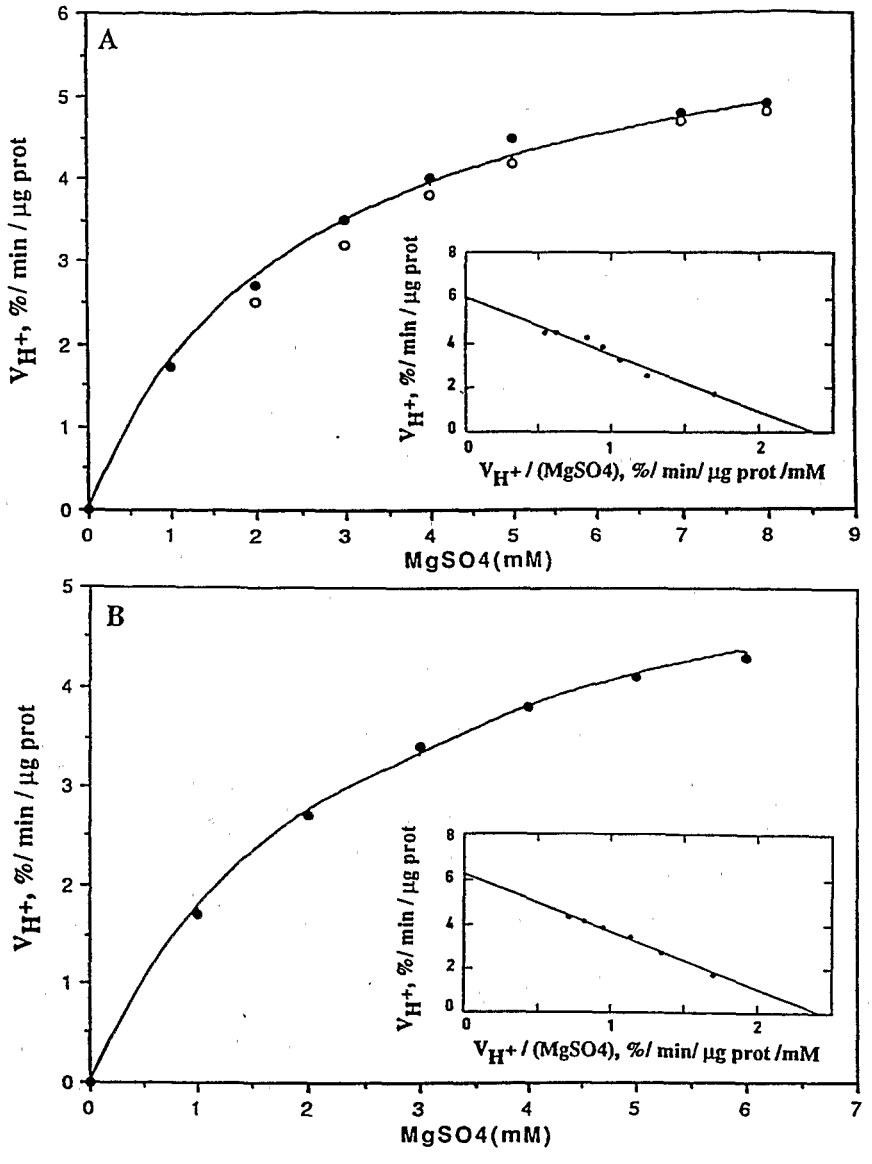

Figure 4. H^+/Mg^{2+} exchange by lutoid vesicles as a function of Mg concentration. A, The acidification reaction resulted from outward Mg^{2+} gradients following EDTA addition to lutoid vesicles loaded with increasing Mg^{2+} concentrations in presence (●) or absence (○) of K^+-valinomycin. Lutoid vesicles were loaded with solution containing 300 mM mannitol, 100 mM K^+ (K_2SO_4), 5 mM Hepes-BTP (pH 8.5), and increasing concentrations of Mg^{2+}. Ten microliters of lutoid vesicles (25 mg/mL of protein) were added to 2 mL of 300 mM mannitol, 100 mM K^+ (K_2SO_4), 5 mM Hepes-BTP (pH 8.5), 1 µM ACMA, and 1 µM valinomycin. V_{H^+} was determined as indicated in "Materials and Methods." Inset, Eadie-Hofstee plot. B, Effect of Mg^{2+} concentration on rate of recovery of fluorescence after an imposed pH difference in the presence of K^+-valinomycin. Lutoid vesicles were loaded with a solution containing 300 mM mannitol, 50 mM K_2SO_4, 5 mM Hepes-BTP (pH 8.5), and 25 mM $(NH_4)_2SO_4$. Ten microliters of lutoid vesicles were added to 2 mL of 300 mM mannitol, 50 mM K_2SO_4, 5 mM Hepes-BTP (pH 8.5), 1 µM valinomycin, and 1 µM ACMA. Aliquots of $MgSO_4$ were added to the cuvette, and the subsequent recovery of fluorescence was followed. Inset, Eadie-Hofstee plot.

Chemicals

ACMA and oxonol VI were purchased from Molecular Probes, Inc. All other chemicals were purchased from Sigma.

RESULTS

In the first experiments, lutoid vesicles or liposomes were loaded with 5 mM Mg^{2+} and added to assay medium containing the same Mg^{2+} concentration. Then, a quasi-infinite outwards diffusion gradient of Mg^{2+} was imposed by adding 5.1 mM EDTA. This resulted in a rapid quenching of the ACMA fluorescence (Fig. 1A). No ACMA quenching was observed with control liposomes, unless the electroneutral H^+/divalent cation exchanger A23187 was present (Fig. 1B). With both kinds of vesicles, the ACMA quenching was reversed by addition of Mg^{2+} (or 0.02% [v/v] Triton X-100 to permeabilize the vesicles, data not shown). We interpret these data as good evidence of H^+/Mg^{2+} antiport activity in lutoid membrane vesicles. Strong electrostatic surface effects (screening and binding effects) of Mg^{2+} have been reported (10) and are well known to modulate quenching of the permeant amine pH dyes, related to their surface stacking (5, 17). We do not believe this is the explanation for our observations, based on the following arguments.

First, similar responses to free Mg^{2+} addition or removal were observed when the ionic strength of the medium was increased by adding 50 mM K_2SO_4 (Fig. 1A, curve c). Second, the ACMA quenching was totally abolished by adding 50 mM $(NH_4)_2SO_4$, due to the neutral diffusion of NH_3 inside the vesicle. Third, although gramicidin did not reverse the ACMA quenching when the medium did not contain K^+, the pH dissipation was observed in the presence of 50 mM K_2SO_4 (Fig. 1A, curve c). These results suggest that a true transmembrane pH gradient was generated by the Mg^{2+} efflux, and that its further dissipation by gramicidin required the presence of K^+ at the exterior to neutralize electrically the passive H^+ efflux.

In a second set of experiments, the effect of externally

added Mg^{2+} on a preformed transmembrane pH gradient was tested with lutoid vesicles and liposomes. Acid-loading (20) was performed by diluting by 200-fold vesicles that had been loaded with 25 mM $(NH_4)_2SO_4$. This acidifies the vesicles' interiors as NH_3 diffuses out of the vesicles. An instantaneous quenching of ACMA fluorescence was observed upon dilution of the vesicles into the assay cuvet (Fig. 2). The ACMA quenching was progressively reversed by increasing Mg^{2+} at the exterior of the lutoid vesicles (Fig. 2A) but not at the exterior of the liposomes, except when the latter contained the ionophore A23187 (Fig. 2B).

The pH optimum of the Mg^{2+}/H^+ antiport was tentatively estimated from the quenching of ACMA by comparing lutoid vesicles and A23187-containing liposomes loaded with 5 mM Mg^{2+} in the same buffer at various pH values (Fig. 3). In these experiments, vesicle acidification was initiated by diluting Mg^{2+}-loaded vesicles into an Mg^{2+}-free solution. The maximum V_H^+ was detected at pH 8.5 with lutoid vesicles (Fig. 3, curve a), whereas the V_H^+ values obtained with liposomes were nearly constant (Fig. 3, curve b).

At pH 8.5, the V_H^+ values versus internal Mg^{2+} concentration were determined using Mg^{2+}-driven proton uptake as described in Figure 1. The V_H^+ values generated by Mg^{2+} efflux followed a saturation curve with a K_m of 2.6 mM and V_{max} of 6% min^{-1} μg^{-1} protein (Fig. 4A). Similar kinetic parameters were determined from the dissipating effect of Mg^{2+} on a preformed pH gradient in acid-loading experiments (Fig. 4B). The observed exchange could be the result of either an Mg^{2+}/H^+ antiporter or an Mg^{2+} uniporter that is electrically coupled to passive H^+ flux. To distinguish between these two possibilities, Mg^{2+}-dependent H^+ flux was examined in the presence of 1 μM valinomycin and symetric levels of potassium (100 mM) on both sides of the membrane vesicle to clamp membrane potential at zero. The K_m and V_{max} values thus obtained were nearly the same as those observed above in the absence of K^+ and valinomycin.

To confirm this point, the membrane potential was estimated using the oxonol VI dye. The response of oxonol VI to an imposed K^+ diffusion potential (positive inside) was first studied in the presence of valinomycin. A high ΔF was observed upon K^+ addition at the exterior, which was totally reversed to F_o by 25 μM SDS (Fig. 5A). The value of the parameter $\Delta F/F_o$ was linear with the membrane potential calculated from the Nernst relation, between 0 and +120 mV (Fig. 5, inset). Creating a zero-trans inward Mg^{2+} diffusion gradient (5 mM at the exterior in the absence of valinomycin) did not induce a change in the oxonol VI fluorescence (Fig. 5B). Thus, the observed Mg^{2+}/H^+ exchange by lutoid vesicles likely resulted from the activation of an electroneutral antiporter $Mg^{2+}/2H^+$.

When lutoid vesicles were incubated for 10 min at pH 8.5 in the presence of increasing concentrations of amiloride or imipramine, which are potent inhibitors of various mono- and divalent cationic antiporters in animal and plant cells, a progressive decrease of V_H^+ and of the maximum ACMA quenching was observed (Figs. 6A and 7A). Half-maximal inhibitions were observed at 0.3 mM amiloride and 0.12 mM imipramine (Figs. 6B and 7B).

DISCUSSION

Several lines of evidence strongly suggest that the lutoidic tonoplast membrane contains an electroneutral $Mg^{2+}/2H^+$ antiporter. The existence of a facilitated transport mechanism for Mg^{2+} is suggested by the Michaelis-Menten kinetics observed for the V_H^+ parameter measured with ACMA as a function of the Mg^{2+} concentration in both influx and efflux experiments (Fig. 4). The K_m value (2.6 mM) observed here is close to that observed for Na^+/Mg^{2+} exchange in erythrocytes (2.6 mM) (8). This K_m value is compatible with the physiological Mg^{2+} concentration reported in serum of latex of the rubber tree (8 mM) (7). However, the significance of this value is obscured for two main reasons. First, the reported Mg^{2+} concentration was for total Mg^{2+}, free plus bound, in the latex serum. Second, the lutoidic vesicle preparation used in the present study should contain approximately 50% inside-out vesicles, as estimated from the latency of the hydrolytic activity of the (H^+)ATPase measured in the presence of Triton X-100 (data not shown). Therefore, the similarity of the K_m values obtained from influx and efflux experiments cannot be taken as definitive evidence for symmetrical facilitated

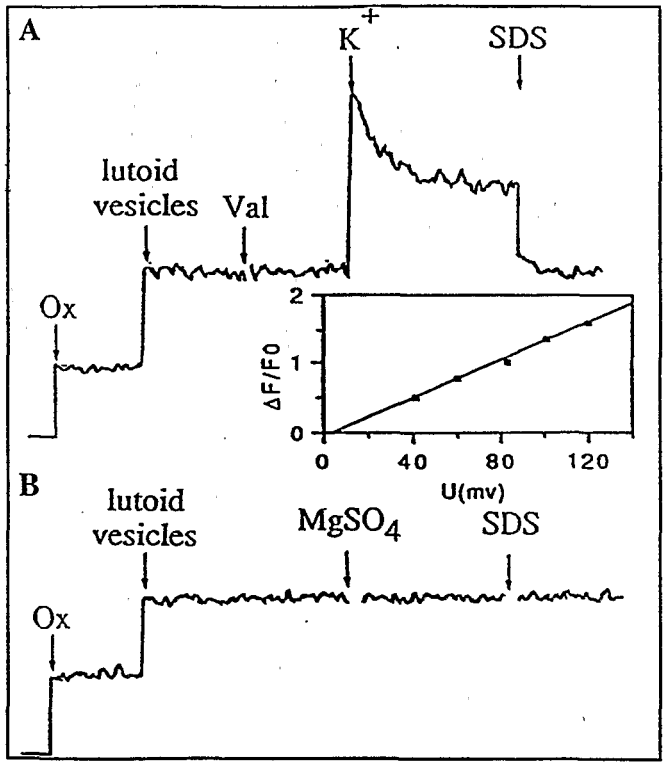

Figure 5. Effect of K^+ and Mg^{2+} inward diffusion gradients on the membrane potential of lutoid vesicles monitored with the oxonol VI dye. A, Membrane potential generated by a K^+ (100 mM) concentration gradient across the lutoid vesicles in the presence of 1 μM valinomycin. Lutoid vesicles were prepared and assayed as described in "Materials and Methods." B, Membrane potential generated by an Mg^{2+} influx across lutoid vesicles. $MgSO_4$ (5 mM) was added after lutoid vesicles were prepared as in A. Inset, Relative fluorescence change, $\Delta F/F_o = (F(U) - F_O)/F_O$, as a function of transmembrane voltage, U.

transport, but merely as the mean "affinity" of both cytoplasmic and intralutoidic faces. The Mg^{2+}/H^+ exchange observed in these experiments seems to result only from the activity of an electroneutral $Mg^{2+}/2H^+$ antiporter, and not from a uniporter, because the K_m and V_{max} parameters were not changed when membrane potential was clamped at zero. Inhibition of the exchange reaction by amiloride and imipramine was observed in the same concentration range as determined for the $Mg^{2+}/2Na^+$ antiporter in animal cells (11). These drugs are well known as potent inhibitors of various monovalent and divalent cation antiporters, generally involving Na^+ in animals (12) and H^+ in plants (2, 3). At pH 8.5, the replacement of Mg^{2+} by Ca^{2+} gave raise to an approximately 6-fold lower acidification rates (data not shown). The apparent optimum pH (8.5) of the lutoidic antiporter obtained from ACMA quenching experiments must be regarded with caution because this value is close to the pK_a of the dye. More accurate experiments, for example using Mg^{2+} fluorescent probes (Mag-Quin 2), will be required. Nevertheless, the $Mg^{2+}/2H^+$ antiporter of the lutoidic membrane seems to exhibit an alkaline optimum pH.

In conclusion, the present study gives the first evidence for the existence in plant cells of an electroneutral $Mg^{2+}/2H^+$ antiporter that is inhibited by amiloride. These properties make this system very different from the Ca^{2+}/H^+ exchange, which is electropositive and insensitive to amiloride (6, 9, 20). The $Mg^{2+}/2H^+$ antiporter of the lutoid membrane might be involved in the regulation of pH and Mg^{2+} concentration in the latex serum. It might also be responsible for the large accumulation of Mg^{2+} in the lutoidic compartment (7).

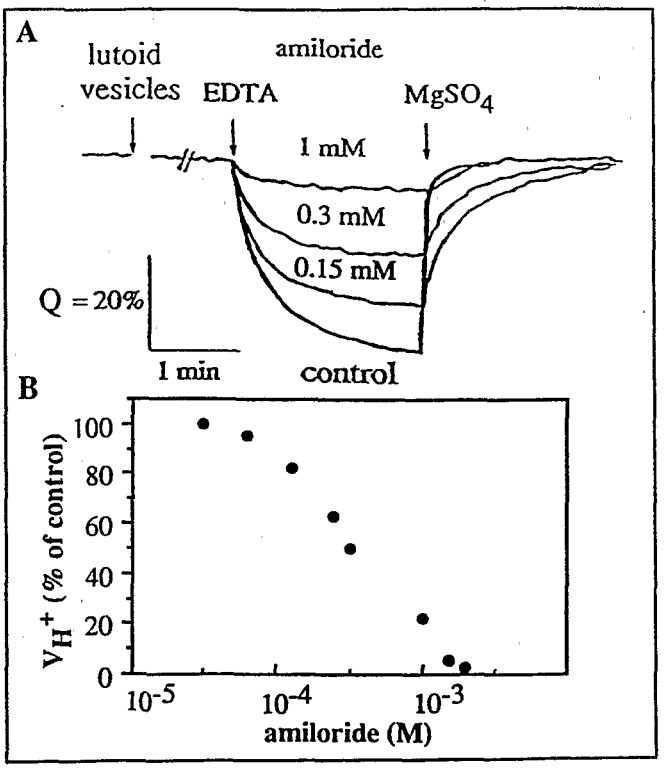

Figure 6. Effects of amiloride on H^+/Mg^{2+} exchange by lutoid vesicles. A, Traces are the quenching of ACMA fluorescence, measured as described in "Materials and Methods." Lutoid vesicles (25 μg/mL of protein) were prepared and assayed as in Figure 1 in the presence of the indicated concentrations of amiloride. B, Plot of the V_H^+ values measured in the presence of increasing amiloride concentrations expressed as percentage of control (V_H^+ = 6% min^{-1} μg^{-1} protein).

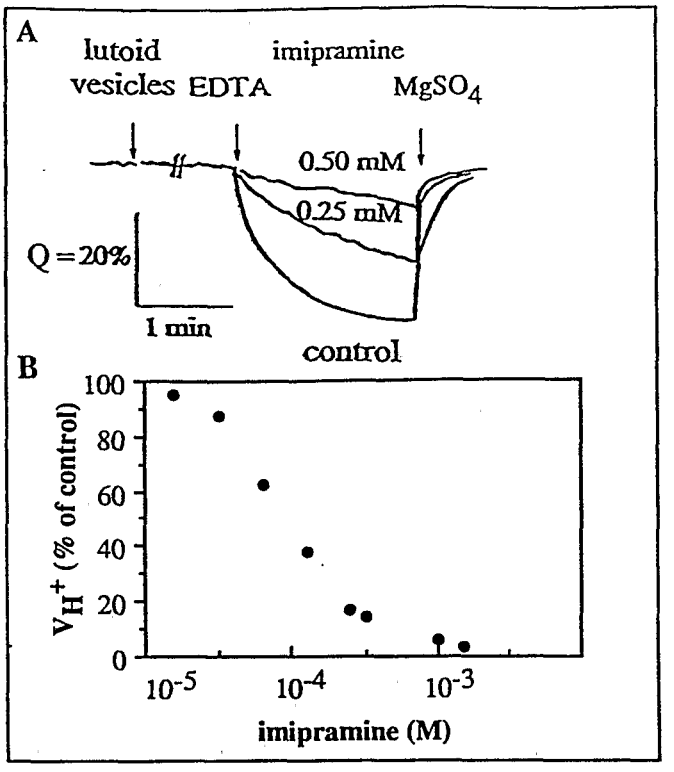

Figure 7. Effects of imipramine on H^+/Mg^{2+} exchange by lutoid vesicles. A, Traces are the quenching of ACMA fluorescence, measured as described in "Materials and Methods." Lutoid vesicles (25 μg/mL of protein) were prepared and assayed as in Figure 1 in the presence of the indicated concentrations of imipramine. B, Plot of the V_H^+ values measured in the presence of increasing imipramine concentrations expressed as percentage of control (V_H^+ = 6% min^{-1} μg^{-1} protein).

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