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Evidence for a Cl^--Stimulated MgATPase Proton Pump in Oat Root Membranes[1]

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ABSTRACT

The possibility that plant membrane-bound MgATPases may act as electrogenic proton pumps has been investigated. Using an oat (*Avena sativa* L. cv. Victory) root membrane preparation which is partially enriched in tightly sealed vesicles, we have shown that MgATP stimulates the uptake of the membrane-permeable anion $[^{14}C]SCN^-$ by the vesicles; this indicates that an electrical potential (interior positive) is generated across the membrane. Both Cl^- ions and the proton ionophore trifluoromethoxy(carbonyl-cyanide)phenylhydrazone inhibit the MgATP-driven $[^{14}C]SCN^-$ uptake, presumably by collapsing the MgATP-generated membrane potential. The uptake of the pH gradient probe $[^{14}C]$imidazole into the vesicles is also greatly stimulated by MgATP, indicating the presence of a transmembrane proton gradient (interior acid). MgATP-driven $[^{14}C]$imidazole uptake is temperature sensitive, Cl^--stimulated, substrate specific for MgATP, sensitive to the MgATPase inhibitors vanadate and *N,N'*-dicyclohexylcarbodiimide, and completely eliminated by trifluoromethoxy(carboxyl-cyanide)phenylhydrazone. The mitochondrial ATPase inhibitor oligomycin has little effect on the MgATPase activity and on the MgATP-dependent $[^{14}C]SCN^-$ and $[^{14}C]$imidazole uptake. These data indicate that a class of oat root membrane-bound MgATPases, stimulated primarily by Cl ions, is capable of using the free energy of ATP-hydrolysis to generate an apparent electrochemical proton gradient *in vitro*.

MgATPases may mediate active proton transport across the plant plasma membrane and tonoplast. Although this idea has gained wide acceptance recently, it is supported primarily by only indirect evidence (19, 21, 26). Plant cells are known to excrete protons, and potential measurements across higher plant cells indicate that electrogenic proton extrusion pumps may drive the active membrane potential across the plasma membrane (21, 26). In several higher plant systems so far examined, the membrane potential is affected by changes in the intracellular ATP level (14, 16). These findings indirectly support the idea that proton extrusion is driven by membrane-bound MgATPases which transduce the free energy of ATP-hydrolysis into a transmembrane electrochemical proton gradient (19, 26). The likelihood that MgATPase proton pumps exist in the plant plasma membrane has also been suggested by the results of investigations of hormone-induced plant cell enlargement (22). It is likely that the active transport of protons occurs also at the tonoplast since, in general, the pH of the vacuole is at least one pH unit lower than the cytoplasm (21). Whether active proton transport at the tonoplast is electrogenic is uncertain, but, as with the proton transport at the plasma membrane, it may serve as the driving force for transmembrane solute movement (19, 26) and may be involved in cytoplasmic pH regulation (21).

Monovalent cation-stimulated MgATPases have been considered to be the most likely candidates for the plasma membrane proton pumps (10, 19) and have been shown to be present in plasma membrane-enriched fractions from a variety of plants (10, 18). ATPases have also been reported to be present in isolated vacuoles and tonoplasts (4, 12, 31). Plant membranes may also contain monovalent anion (Cl^-)-stimulated MgATPases (10), which may be located in the tonoplast (10) as well as the plasma membrane (2, 9). It is possible that these anion-stimulated MgATPases may also be involved in the generation of transmembrane pH gradients in plant cells.

If plant membrane MgATPases are involved in the electrogenic transport of H ions (or OH ions), it should be possible to demonstrate *in vitro* MgATP-driven electrochemical proton gradients across plant membrane vesicles. Attempts to demonstrate net proton transport using such an approach have generally been unsuccessful using higher plant membrane preparations, probably because most of the vesicles were leaky to protons as well as to other ions (30). Sze (28) has recently described a procedure for obtaining a plant membrane fraction partially enriched in tightly sealed vesicles. This conclusion was based in part on the low K^+ permeability coefficient of a large portion (about 40%) of the membrane vesicle population (28).

We have used this technique to isolate membrane vesicles from oat roots and have used them to demonstrate ATP-driven electrogenic proton transport *in vitro*. In this report, we present direct evidence in favor of the idea that plant membrane MgATPases can function as electrogenic proton pumps.

MATERIALS AND METHODS

Plant Material. Seeds of *Avena sativa* L. cv. Victory were germinated and grown on moist vermiculite as previously described (27). Intact 5-d-old seedlings were gently removed, and the roots were washed in distilled H_2O to remove the vermiculite. Whole roots (5–10 cm long) were excised and washed twice in ice-cold distilled H_2O prior to homogenization.

Membrane Vesicle Preparation. Preparation of membrane vesicles was performed as described by Sze (28). In the method used here, mannitol has been substituted for sucrose, and the protease inhibitor PMSF[2] has been included in the grinding medium. The excised oat roots were chopped up with a razor blade and then homogenized using an ice-cold mortar and pestle. All subsequent procedures were carried out at 0 to 5°C. The homogenization

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[2] Abbreviations: PMSF, phenylmethylsulfonylfluoride; EGTA, ethylene glycol-bis(β-aminoethyl)-*N,N'*-tetraacetic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DCCD, *N,N'*-dicyclo-hexylcarbodiimide; FCCP, trifluoromethoxy(carbonyl-cyanide)phenylhydrazone.

medium consisted of 260 mM mannitol, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 25 mM Tris-HCl (pH 7.3), with about 4 ml homogenization medium/g fresh weight of tissue. The homogenate was strained through two layers of Miracloth (Calbiochem) and was centrifuged at 13,000*g* for 15 min. The 13,000*g* supernatant was centrifuged at 80,000*g* for 45 min to obtain a crude microsomal pellet which was resuspended with 260 mM mannitol in 2.5 mM Tris-HCl (pH 7.4).

An 8-ml volume of 10% Dextran T70 (w/w) in 260 mM mannitol buffered with 2.5 mM Tris-HCl (pH 7.4) was pipetted into a 13-ml polyallomer centrifuge tube, and then the crude microsomal suspension (about 5 ml) was gently layered on top of the Dextran cushion. This was centrifuged in a swinging bucket rotor (SW-27) for 2 h at 20,000 rpm (about 70,000*g*-max). After centrifugation, a membrane band visible at the mannitol/Dextran interface was removed with a Pasteur pipet. This membrane fraction (about 1.5 ml; 1–2 mg protein) is referred to as interface vesicles (28).

ATPase Assay. ATPase activity was assayed at 25°C as previously described (27). The final concentrations of the components of a typical assay medium were 33 mM Mes- or Pipes-Tris (pH 6.7), 3 mM $MgSO_4$, 3 mM ATP-Tris (pH 6.7), and 50 mM KCl when present. The Pi produced during ATP hydrolysis was estimated by the method of Fiske and Subbarow (6). KCl-stimulated MgATPase (KCl-MgATPase) activity is defined here as the difference in activities with and without 50 mM KCl. Cl^--stimulated MgATPase (Cl^--MgATPase) activity is the difference in activity in the presence and absence of 50 mM choline chloride.

Protein Determination. Protein was estimated by the method of Lowry *et al.* (13). Because of interference by the Dextran T70, protein was first precipitated with ice-cold 12.5% TCA. This was pelleted by centrifugation at about 5,000*g* for 10 min, and then the pellet was dissolved in a volume of 1% SDS in 0.1 N NaOH. BSA was used as a standard.

[^{14}C]Thiocyanate and [^{14}C]Imidazole Uptake Assay. The membrane permeable anion [^{14}C]thiocyanate ([^{14}C]SCN^-) was used to monitor the formation of electrical potentials across these interface membrane vesicles, because SCN^- will become asymmetrically distributed across membrane vesicles in the presence of a membrane potential (24). [^{14}C]Imidazole has previously been used to monitor ATP-generated pH gradients in membrane vesicles from mucosa (23) and *Neurospora* plasma membranes (25). Because the protonated form of this weak base is less permeable to membranes than the neutral form (pK = 7), the charged form of imidazole will accumulate on the acid side of a membrane in the presence of a transmembrane pH gradient (25).

To determine the uptake of [^{14}C]SCN^- or [^{14}C]imidazole by the oat root interphase vesicles, we employed a modified version of a Millipore filtration technique described by Scarborough (24). The final concentrations of the basic components of the uptake assay medium were 200 to 220 mM mannitol, 10 mM Mes-Tris or Pipes-Tris (pH 6.7), 0.5 mM EGTA, 2.5 or 5.0 mM $MgSO_4$, 5 μg oligomycin (final concentration of ethanol = 0.1%), 50 mM KCl when present, 2.5 or 5.0 mM ADP- or ATP-Tris (pH 6.7), and 50 μM K[^{14}C]SCN or [^{14}C]imidazole. To initiate the reaction, 50 to 100 μl of the freshly isolated interphase membranes (50–200 μg protein) were added to a test tube containing the above medium and various additions (see figure legends) to yield a final volume of 0.5 ml. The samples were incubated at 25°C for the desired time intervals. About 20 s prior to termination, the assay medium was taken up into a Pasteur pipet. The reaction was stopped by adding the medium dropwise to the center of a 25 mm Millipore 0.45 μM HATF filter (prewet with distilled H_2O), and then the filter was immediately rinsed with 2 ml of ice-cold 260 mM mannitol in 10 mM Mes-Tris (pH 6.7). The filtration time was usually 10 to 20 s and the rinse time was usually less than 10 s. This termination technique provided much better replicates in our hands than did the dilution method (24, 25). Zero time controls were determined by filtering the assay medium (-ATP) immediately after adding the membranes, using the termination procedure described above. The amount of radioactivity adhering to the Millipore filter alone was determined by filtering the assay medium, minus the membranes, using the above procedure and was subtracted from all the experimental values, including the zero time value. The filters were immediately transferred to scintillation vials containing 5 ml Aquasol (New England Nuclear), and the radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer.

Materials. Oligomycin, sodium azide, Triton X-100, PMSF, EGTA, and DCCD were purchased from Sigma. FCCP was from DuPont. Dextran T70 was obtained from Pharmacia. 5′-Nucleotides were all sodium salts obtained from Sigma and were converted to the Tris-salts (pH 6.7) by Dowex 50 ion exchange. Sodium orthovanadate was from Fisher. All other chemicals were reagent grade.

K[^{14}C]SCN (62.0 mCi/mmol) was purchased from Amersham/Searle, and [^{14}C]imidazole (1.32 mCi/mmol) was from California Bionuclear Corp., Sun Valley, CA.

RESULTS

MgATPase Studies. In preliminary experiments, we found that the proton ionophore FCCP increased the KCl-stimulated MgATPase activity in our oat root interface vesicles by 20 to 50% (data not shown). This ionophore stimulation of MgATPase activity agreed with results reported by Sze (28). FCCP will facilitate the transport of protons across lipid bilayer membranes and will collapse an ATP-driven transmembrane ΔpH. Such a breakdown of the electrochemical proton gradient might be expected to stimulate the activity of proton-transport MgATPases in tightly sealed vesicles by relieving an electrical and/or pH backpressure on the pump (28). This preliminary result suggested that we had some tightly sealed vesicles in our membrane preparation which contained proton-transport MgATPases.

The possibility that interphase vesicles from oat roots contain significant amounts of mitochondrial ATPases have been eliminated in three ways. First, oligomycin and sodium azide, inhibitors of mitochondrial ATPase activity (17), inhibited oat root interphase vesicle KCl-MgATPase activity by only 10 to 15% at both pH 6.7 and 8.5 (Table I). Secondly, the pH optimum for the KCl-MgATPase activity was about pH 7.0 in the presence of FCCP. If mitochondrial ATPases were a significant contaminant of this membrane fraction, then we would have expected a pH optimum of about pH 9.0 as reported for oat root mitochondrial ATPases

Table I. *Effect of Inhibitors on KCl-Stimulated MgATPase Activity of Oat Root Interface Vesicles*

ATPase activity was assayed as described in "Materials and Methods" at 25°C in a medium consisting of 3 mM ATP-Tris, 33 mM Mes-Tris (pH 6.7 and 8.5, respectively) and 3 mM $MgSO_4$, in the presence and absence of 50 mM KCl, 10 μg/ml oligomycin, 100 μM sodium orthovanadate, and 0.5 mM sodium azide. All samples contained 0.1% ethanol. Reactions were started by adding membranes (about 30 μg protein).

Treatment	KCl-MgATPase Activity	
	pH 6.7	pH 8.5
	nmol Pi/mg protein·min	
Control	76 (100)[a]	60 (100)
+Oligomycin	69 (91)	51 (85)
+Azide	64 (84)	50 (84)
+Vanadate	30 (39)	27 (45)
+Vanadate, +oligomycin	27 (30)	21 (35)

[a] The numbers in parentheses indicate the percentage of the control activity.

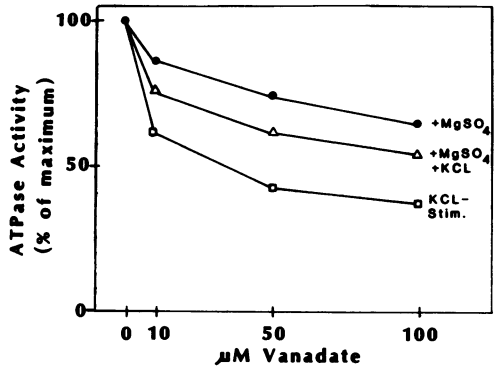

FIG. 1. ATPase activity of oat root interface membranes as a function of sodium vanadate concentration. ATPase activity was assayed as described in "Materials and Methods" in the presence and absence of 50 mM KCl and sodium vanadate (at the concentrations shown). KCl-stimulated MgATPase activity is the difference in activity in the presence and absence of KCl. Maximum activities were 143 (+$MgSO_4$), 230 (+$MgSO_4$, +KCl), and 87 (KCl-stimulated) nmol Pi/mg protein·min.

Table II. *Effect of Various Potassium and Chloride Salts on MgATPase Activity in the Presence and Absence of FCCP and Triton X-100*

ATPase activity was determined as previously described at 25°C in a medium consisting of 33 mM Pipes-Tris (pH 6.7), 3 mM $MgSO_4$, 5 μg oligomycin (10 μg/ml), 3 mM ATP-Tris, and in the presence and absence of the salts listed below and 5 μM FCCP or 0.01% Triton X-100. All samples contained 0.5% ethanol.

Treatment	MgATPase Activity		
	Control	+FCCP	+Triton
	nmol/mg protein·min		
	160 (100)[a]	212 (100)	232 (100)
KCl, 50 mM	217 (136)	293 (138)	306 (132)
K_2SO_4, 25 mM	172 (108)	244 (115)	240 (103)
Choline Cl, 50 mM	206 (129)	276 (130)	310 (134)
Tris Cl, 50 mM	219 (137)	276 (130)	305 (132)

[a] The numbers in parentheses indicate the percentage of the activity in the absence of added salts.

(11). Finally, as also shown in Table I, 100 μM vanadate inhibits about 60% of the KCl-MgATPase activity in oat root interface vesicles. Vanadate has recently been shown to have little or no effect on mitochondrial ATPase activity from *Neurospora* (1) and higher plants (3), while it is a powerful inhibitor of the *Neurospora* plasma membrane MgATPase (1) as well as membrane-bound MgATPases of higher plant tissues (3, 18). Figure 1 illustrates the sensitivity of the ATPase activities in oat root interface vesicles to increasing concentrations of vanadate and shows that the KCl-MgATPase activity was the most sensitive to vanadate. This level of sensitivity to vanadate is very similar to that of the plasma membrane KCl-stimulated MgATPase activity of corn leaves (18). The results of Table I and Figure 1 indicate that the bulk (about 60%) of the KCl-MgATPase activity at pH 6.7 in oat root interface vesicles is vanadate sensitive, that about 10 to 15% is probably due to mitochondrial ATPases, and that the remaining 25 to 30% is due to vanadate/oligomycin-insensitive ATPases and/or nonspecific phosphatases. To suppress the small amount of contaminating mitochondrial ATPase activity at pH 6.7, we routinely included oligomycin (50–100 μg/mg protein) in all assay media.

During the course of our studies, some questions arose regarding the ion-sensitivity of the MgATPase activity present in the oat root interface vesicles. Table II shows an example of the effects of potassium and chloride salts on the pH 6.7 MgATPase activity in our interface membranes. Cl ions stimulated the MgATPase activity much more than K ions. This Cl^--stimulation was apparent in both the presence and absence of 0.01% Triton X-100 and 5 μM FCCP which would be expected to prevent the formation of a MgATP-driven electrical potential across tightly sealed vesicles. This indicated that the Cl^--stimulation was due to a direct effect on the MgATPase activity rather than to a possible indirect effect via the relief of a membrane potential-induced backpressure on ion-transport MgATPases. We found that, on average, Cl^--stimulation of the pH 6.7 MgATPase activity in oat root interface vesicles was 4 to 5 times greater than the K^+-stimulation. Thus, the interface fraction appeared to be enriched in Cl^--stimulated

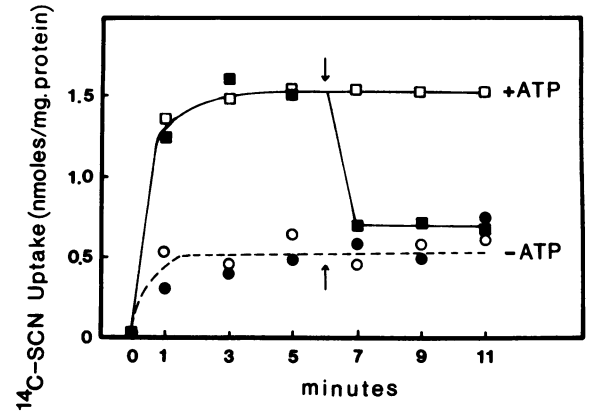

FIG. 2. Time course of [^{14}C]SCN^- uptake by oat root interface vesicles. [^{14}C]SCN^- uptake was assayed at 25°C in the presence of 200 mM mannitol, 10 mM Pipes-Tris (pH 6.7), 0.5 mM EGTA, 5 mM $MgSO_4$, 5 μg oligomycin (10 μg/ml), 50 μM K[^{14}C]SCN, and in the presence (□, ■) and absence (○, ●) of ATP-Tris (pH 6.7). Reactions were started by adding membranes (about 0.5 mg protein total) to yield a final volume of 3.5 ml. At the time points indicated, 0.5-ml aliquots were removed and immediately filtered to determine the [^{14}C]SCN^- uptake. At 6 min after adding the membranes, 10 μl of either 100% ethanol (□, ○) or 1 mM FCCP (■, ●), in 100% ethanol, were added to the reaction medium, as marked by the arrows. Final concentrations of ethanol and FCCP were about 0.5% and 5 μM, respectively. In this experiment, the zero time value equaled about 0.6 nmol [^{14}C]SCN^-/mg protein and was subtracted from the [^{14}C] SCN^- uptake value from all subsequent time points.

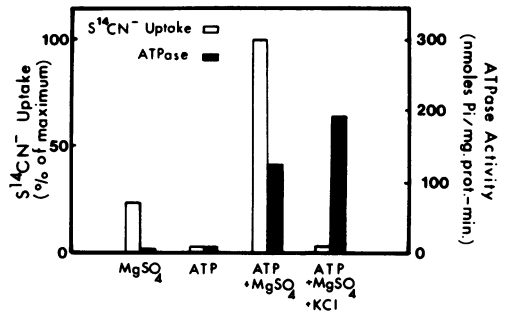

FIG. 3. [^{14}C]SCN^- uptake and ATPase activity of oat root interphase vesicles under various conditions. [^{14}C]SCN^- uptake was assayed in a medium consisting of 200 mM mannitol, 10 mM Mes-Tris (pH 6.7), 0.5 mM EGTA, 5 μg oligomycin (10 μg/ml), 50 μM K[^{14}C]SCN, and in the presence and absence of 5 mM $MgSO_4$, 5 mM ATP-Tris (pH 6.7), and 50 mM KCl. Reactions were started by adding membranes (about 75 μg protein) to yield a final volume of 0.5 ml and were terminated after 10 min at 25°C. ATPase activity was assayed at 25°C in the presence of 33 mM Mes-Tris (pH 6.7), 5 μg oligomycin (0.1% ethanol in all samples), and in the presence and absence of 3 mM $MgSO_4$, 3 mM ATP-Tris (pH 6.7), and 50 mM KCl. All the [^{14}C]SCN^- uptake values reported here were corrected by subtracting the zero time value (0.8 nmol/mg protein) for this experiment. Maximum [^{14}C]SCN^- uptake was 1.5 nmol/mg protein.

MgATPase (Cl^--MgATPase) activity. It should be emphasized here that the KCl-MgATPase activity referred to in this report is stimulated much more by Cl ions than by K ions.

[^{14}C]SCN$^-$ Uptake Studies. We used [^{14}C]SCN$^-$ uptake by the oat root interface vesicles to determine whether a MgATP-driven membrane potential was generated in these vesicles. Figure 2 shows that in the absence of ATP the [^{14}C]SCN$^-$ uptake was minimal, probably representing the equilibrium partitioning of the radioiosotope between the inside and outside of the vesicles (24). In the presence of MgATP, there was a marked increase in [^{14}C]SCN$^-$ uptake within 1 min which reached equilibrium 3 to 5 min after the vesicles are added. This result indicated that the interior of at least some of the membrane vesicles became positive (+) in the presence of MgATP. The amount of MgATP-dependent [^{14}C]SCN$^-$ uptake ranged from 0.5 to 1.8 nmol SCN^-/mg protein over the course of our experiments. We attributed this to variability among the different membrane preparations used. Figure 2 also shows that FCCP almost completely collapsed the MgATP-driven [^{14}C]SCN$^-$ uptake without affecting the control level of uptake. This supports the idea that the MgATP-dependent [^{14}C]-SCN$^-$ uptake we observed was due to a membrane potential-induced accumulation of the radioisotope inside tightly sealed vesicles, rather than to increased [^{14}C]SCN$^-$ binding to the membranes. The collapse of this apparent MgATP-driven membrane potential by FCCP was also indirect evidence that protons were the ions that were actively accumulated in these vesicles. These results agree with the recently reported findings of Rasi-Caldogno *et al.* (20) and Sze and Churchill (29).

The rate of ATP-hydrolysis by plant MgATPases is dependent upon the presence of Mg ions and is stimulated by the presence of KCl (10, 11). Figure 3 indicates that this ATP-stimulated [^{14}C]-SCN$^-$ uptake was dependent on the presence of $MgSO_4$, inasmuch as ATP alone had no significant effect on the uptake. Thus, the formation of a membrane potential does not appear to occur in the presence of ATP unless significant amounts of ATP-hydrolysis can occur (+Mg, +ATP). Figure 3 also shows that even though 50 mM KCl stimulates ATPase activity, KCl apparently collapses the MgATP-driven membrane potential in these vesicles. Influx of Cl ions probably acts to dissipate the MgATP-generated membrane potential since both sodium chloride and choline chloride also decrease the MgATP-driven [^{14}C]SCN$^-$ uptake (data not shown).

[^{14}C]Imidazole Uptake Studies. If active proton transport is chiefly responsible for the MgATP-driven membrane potential in these membrane vesicles, then a detectable MgATP-generated transmembrane pH gradient should exist. We have measured the uptake of the pH probe [^{14}C]imidazole by the interphase vesicles under various conditions in an attempt to detect *in vitro* transmembrane proton translocation by oat root MgATPases.

As shown in Figure 4, the addition of MgATP greatly stimulated the uptake of [^{14}C]imidazole by the interface vesicles over the control level (-ATP). This MgATP-stimulated [^{14}C]imidazole uptake indicated that MgATP drives the net accumulation of protons inside at least some of the membrane vesicles. Under the conditions used here, the typical half-time for MgATP-driven [^{14}C]-imidazole uptake was 0.5 to 1.0 min, reaching equilibrium within 2 to 3 min. This equilibrium was maintained for up to 15 min, and further addition of ATP did not increase the uptake of [^{14}C]imidazole beyond this level (data not shown). During our studies, MgATP-driven [^{14}C]imidazole uptake ranged from 0.8 to 2.3 nmol imidazole/mg protein. As expected, FCCP completely eliminated the MgATP-dependent [^{14}C]imidazole uptake, presumably by increasing the proton conductance of the tightly sealed vesicles.

To ensure that these observations were a result of MgATPase activity, we examined the effects of several factors which influence the rate of ATP-hydrolysis by plant MgATPases on MgATP-stimulated [^{14}C]imidazole uptake.

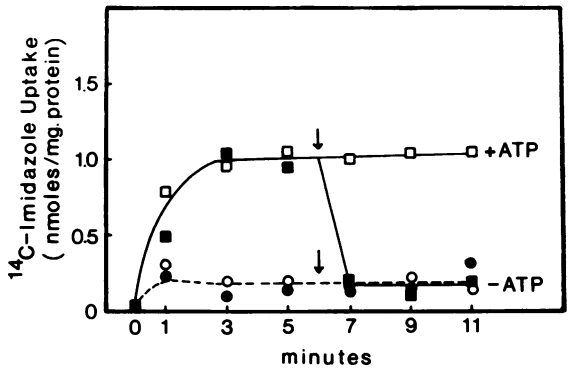

FIG. 4. Time course of [^{14}C]imidazole uptake by oat root interface vesicles. The [^{14}C]imidazole uptake was assayed at 25°C using the procedure described in Figure 2 except that 50 μM [^{14}C]imidazole was used instead of K[^{14}C]SCN and 50 mM KCl was included in the assay medium. (Membrane protein/0.5-ml aliquot = about 100 μg.) Zero time value = about 1.3 nmol [^{14}C]imidazole/mg protein. The 10 μl of either 1 mM FCCP or 100% ethanol were added at the arrow. +ATP (□, ■); -ATP (○, ●); +ethanol (□, ○); +FCCP (■, ●).

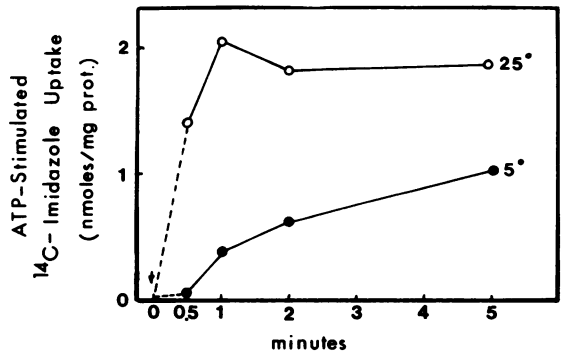

FIG. 5. Time course of ATP-stimulated [^{14}C]imidazole uptake by vesicles at 25 or 5°C. [^{14}C]Imidazole uptake was assayed at either 5 or 25°C in the presence of 200 mM mannitol, 10 mM Pipes-Tris (pH 6.7), 0.5 mM EGTA, 5 μg oligomycin, 2.5 mM $MgSO_4$, 50 mM KCl, 50 μM [^{14}C]imidazole, and 2.5 mM ADP- or ATP-Tris (pH 6.7). Membranes (about 160 μg protein/sample) were preincubated in the assay medium (minus ADP and ATP) for 2 min, and the reaction was started by adding either ADP or ATP and terminated at the times shown. MgATP-stimulated uptake is the difference in [^{14}C]imidazole uptake in tbe presence and absence of ATP. The KCl-MgATPase activities at 5 and 25°C were 10 and 42 nmol Pi/mg protein·min, respectively.

Low temperature inhibited both MgATPase activity and MgATP-stimulated [^{14}C]imidazole uptake as indicated in Figure 5. The permeability of the membranes to the [^{14}C]imidazole appeared to be slightly decreased (15–20%) by the lower temperature, but this is not enough to account for the observed differences in MgATP-stimulated uptake of [^{14}C]imidazole at 25°C *versus* 5°C.

Table III shows that ATP-stimulated [^{14}C]imidazole uptake was dependent on the presence of $MgSO_4$, stimulated by both KCl and choline chloride, and unaffected by K_2SO_4. As also shown in Table III, the Cl^- stimulation of the MgATP-driven [^{14}C]imidazole uptake was not affected by the presence of 0.1 mM KSCN which should collapse the MgATP-generated membrane potential in the sealed vesicles. This is indirect evidence that Cl^- increases the MgATP-driven transmembrane proton gradient in these vesicles by directly stimulating the MgATPase activity, not by collapsing the membrane potential.

Plant membrane-bound MgATPases are quite substrate specific for ATP (10, 18). Figure 6 shows that [^{14}C]imidazole uptake and

Table III. *Effect of Various Salts on the ATP-Dependent [^{14}C]Imidazole Uptake*

[^{14}C]Imidazole uptake was assayed at 25°C in the presence of 220 mM mannitol, 10 mM Mes-Tris (pH 6.7), 0.5 mM EGTA, 5 μg oligomycin (10 μg/ml), and in the presence and absence of 2.5 mM $MgSO_4$, 50 mM KCl or choline chloride, 25 mM K_2SO_4, 0.1 mM KSCN, and 2.5 mM ATP-Tris (pH 6.7). Reactions were started by adding membranes and terminated after 10 min. ATP-dependent [^{14}C]imidazole uptake is defined here as the difference in uptake in the presence and absence of ATP.

Treatment	ATP-Dependent [^{14}C]Imidazole Uptake
	nmol/mg protein
$-MgSO_4$	0
$+MgSO_4$	0.5
$+MgSO_4$, +KCl	2.3
$+MgSO_4$, $+K_2SO_4$	0.3
$+MgSO_4$, +Choline Cl	2.2
$+MgSO_4$, +KSCN	0.3
$+MgSO_4$, +KCl, +KSCN	2.2
$+MgSO_4$, +Choline Cl, +KSCN	2.2

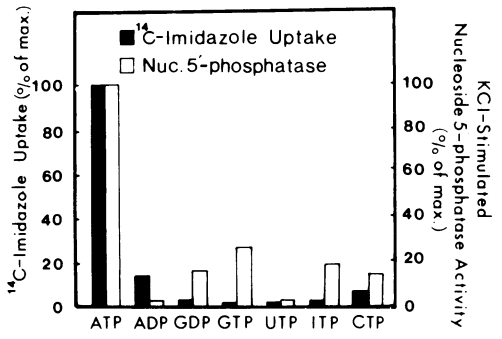

FIG. 6. Substrate specificity of [^{14}C]imidazole uptake and KCl-Mg-ATPase activity of oat root interphase vesicles. [^{14}C]Imidazole uptake was determined as described in Table III. The concentration of the 5'-nucleoside phosphates tested was 2.5 mM. The KCl-stimulated 5'-nucleoside phosphatase activities were determined at 25°C using the procedure for measuring KCl-MgATPase activity as previously described (100% activity = 68 nmol Pi/mg protein·min). The 100% level of [^{14}C]imidazole uptake = 1.9 nmol/mg protein (zero time value [1.7 nmol/mg protein] subtracted).

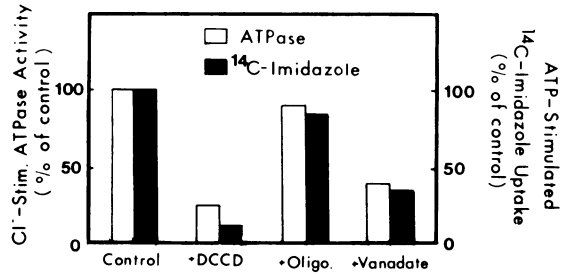

FIG. 7. Effects of ATPase inhibitors on MgATP-stimulated [^{14}C]imidazole uptake and Cl^--MgATPase activity. [^{14}C]Imidazole uptake was assayed essentially as described in Figure 6, and Cl^--MgATPase activity was determined as described using 50 mM choline chloride instead of 50 mM KCl. Both [^{14}C]imidazole uptake and Cl^--MgATPase activity were measured in the presence and absence of 10 μM DCCD, 5 μg oligomycin (10 μg/ml), and 100 μM vanadate. Control Cl^--MgATPase activity = 66 nmol Pi/mg protein·min. Control MgATP-stimulated [^{14}C]imidazole uptake = 2.1 nmol/mg protein.

5'-nucleoside phosphatase activity in these oat root interphase membrane vesicles is also apparently specific for ATP.

A typical example of the effects of vanadate, the MgATPase inhibitor DCCD, and the mitochondrial ATPase inhibitor oligomycin on MgATP-driven [^{14}C]imidazole uptake and Cl^--Mg-ATPase activity in our oat root interface membranes is shown in Figure 7. DCCD and vanadate inhibited the MgATP-dependent [^{14}C]imidazole uptake by 80% and 60%, respectively. Oligomycin inhibited the uptake by about 10%. These values are comparable to the degree of inhibition of Cl^--MgATPase activity by each of these inhibitors. None of these inhibitors significantly affected the level of [^{14}C]imidazole uptake in the absence of ATP.

DISCUSSION

We have demonstrated directly, using [^{14}C]imidazole uptake, that a MgATP-driven transmembrane pH gradient can be detected in isolated oat root membrane vesicles. The formation of this pH gradient is specific for MgATP, stimulated by Cl^-, and significantly reduced by MgATPase inhibitors DCCD and vanadate. Using [^{14}C]SCN^-, we have also monitored *in vitro* the formation of a MgATP-driven membrane potential. This potential gradient is probably a result of active proton transport across the membrane, since it is dissipated by the protonophore FCCP. However, our results do not rule out the possibility that the active transport of other ions may also contribute to the observed MgATP-driven membrane potential. Since Gross and Marme (7) have reported MgATP-driven Ca^{2+} uptake into plant membrane vesicles, we included EGTA in our assay medium (25) to remove Ca^{2+} from consideration in the transport experiments reported here. The small effects of the mitochondrial ATPase inhibitor oligomycin on the MgATPase activity and on the MgATP-dependent uptake of [^{14}C]SCN^- and [^{14}C]imidazole in these oat root interface vesicles argue against the involvement of mitochondrial ATPases in our results. Taken together, the evidence indicates that a class of oat root membrane-bound MgATPases can mediate the electrogenic transport of protons into tightly sealed vesicles.

The magnitude of the MgATP-generated membrane potential and the pH gradient cannot be accurately calculated from these data because we lack information regarding the proportion and volume of active vesicles in this membrane preparation. It is assumed that only a minority of the total vesicle population is of this type since probably less than half of the vesicle population is tightly sealed (28) and since only a fraction (*e.g.* 50%) of the tightly sealed vesicles would be expected to contain the active MgATPases exposed to the external medium.

The MgATPase activity present in the oat root interface vesicles is stimulated more by Cl ions than by monovalent cations. On average, the basal MgATPase activity at pH 6.7 was stimulated about 40% by Cl^- and about 10% by K^+. FCCP, which dissipated the proton gradient, stimulated the MgATPase activity, presumably by eliminating an electrical or pH backpressure on the MgATPase (28). Since Cl ions dissipated the MgATP-driven membrane potential, we presumed that Cl^- may stimulate the MgATPase activity by eliminating an electrical backpressure on the pump. However, upon closer examination of the Cl^- effect, we found what appears to be a direct effect of Cl ions on the MgATPase activity, since Cl^- stimulates the MgATPase activity in both the presence and absence of an electrical potential across the active vesicles. Since the oat root interface vesicles are enriched in Cl^--MgATPase, rather than K^+-MgATPase, activity, what happened to the K^+-MgATPase activity which is known to occur in oat root membranes (10, 11)? In preliminary experiments, we have found that the bulk of the total K^+-MgATPase activity (stimulated by 25 mM K_2SO_4) is present in the membrane pellet at the bottom of the Dextran T70 cushion, while only a small fraction (about 5 to 10%) is present in the interface fraction.

Several lines of evidence indicate that the Cl^--stimulated

MgATPase, rather than the K^+-stimulated MgATPase, is primarily responsible for the MgATP-driven transmembrane pH gradients that we have measured. Most of the MgATPase activity in this membrane preparation is stimulated by Cl ions and not by K ions. Potassium ions had no observable effect on MgATP-dependent [^{14}C]SCN^- or [^{14}C]imidazole uptake by the vesicles. Although MgATP-dependent [^{14}C]imidazole uptake did occur in the absence of Cl ions, both KCl and choline Cl stimulated the MgATP-generated ΔpH *in vitro* in both the presence and absence of thiocyanate (SCN^-) which ensured that the Cl^--stimulation was not due to a neutralization of the MgATP-generated membrane potential. Both the Cl^--MgATPase activity and the MgATP-driven [^{14}C]imidazole uptake in oat root interphase vesicles are inhibited about 60% by 100 μM sodium vanadate, and both are equally sensitive to oligomycin and DCCD. These data provide strong, albeit indirect, evidence that the Cl^--stimulated MgATPases mediate the MgATP-generated electrochemical proton gradient in these vesicles.

The identity of the membranes from which the active vesicles are derived is not known for certain. The interface membrane preparation probably consists of a mixture of membrane types, although it appears to contain a relatively small amount of mitochondrial membranes. K^+-stimulated MgATPase activity has been used as a qualitative marker for plant plasma membranes (10, 18); however, it has been proposed that Cl^--stimulated MgATPases may reside in the plant plasma membrane (4, 9) as well as the tonoplast (10). Unfortunately, there are many reports in the literature of plant membrane-bound K^+-stimulated MgATPases in which the chloride salt of potassium is used without any consideration of the anion effect on the MgATPase. It is possible that Cl^--MgATPases may also become a marker for the plasma membrane in certain plants. The vanadate sensitivity of the Cl^--stimulated component of our oat root interface MgATPase activity suggests a plasma membrane origin for this MgATPase since the plasma membrane KCl-stimulated MgATPase of corn leaves (18) and the plasma membrane MgATPase proton pump of *Neurospora* (1) are both specifically inhibited by vanadate. At this time, it is not known for certain whether or not tonoplast MgATPases are inhibited by vanadate because of conflicting data (4, 31). However, several recent reports have appeared of vanadate-insensitive MgATP-dependent proton pumping in plant membrane vesicles (5, 15) which suggests that the vesicles which are monitored may have a tonoplast origin. Because of lack of definite information regarding the ion-stimulation and inhibitor sensitivity of higher plant plasma membrane and tonoplast MgATPases, we can not determine for certain whether the MgATP-generated ΔpH we have monitored occurs chiefly in inside-out plasma membrane vesicles or right-side-out tonoplast vesicles. The possibility that membrane vesicles of other origin are contributing to the observed MgATP-driven membrane potential and pH gradient also must be considered, however, since ATP-driven proton pumping into ER and golgi membrane vesicles of corn coleoptiles has recently been reported (8).

An important goal for future studies will be to determine the identity of the active vesicles, by using membrane fractionation techniques to separate the various membrane types and, subsequently, by using enzyme and hormone-binding markers to identify the membranes. We feel that this study and other recent reports of MgATP-driven proton transport in interface membrane vesicles (5, 15, 29) indicate that more attention should be focused on monovalent anion (Cl^-)-stimulated MgATPases in higher plants, since their existence in plant membranes and their role in plant membrane transport has largely been neglected.

In conclusion, these results provide direct evidence that Cl^--stimulated MgATPase electrogenic H^+ (or OH^-) pumps exist in oat root membranes. At the present time, the exact identity of the active vesicles is unknown, although we suspect that they originate from the tonoplast and/or the plasma membrane. We are convinced that mitochondrial membrane ATPases are not a contributing factor. Most importantly, we feel that the results presented here support the hypothesis that membrane-bound MgATPases from higher plants can function as electrogenic proton pumps.

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